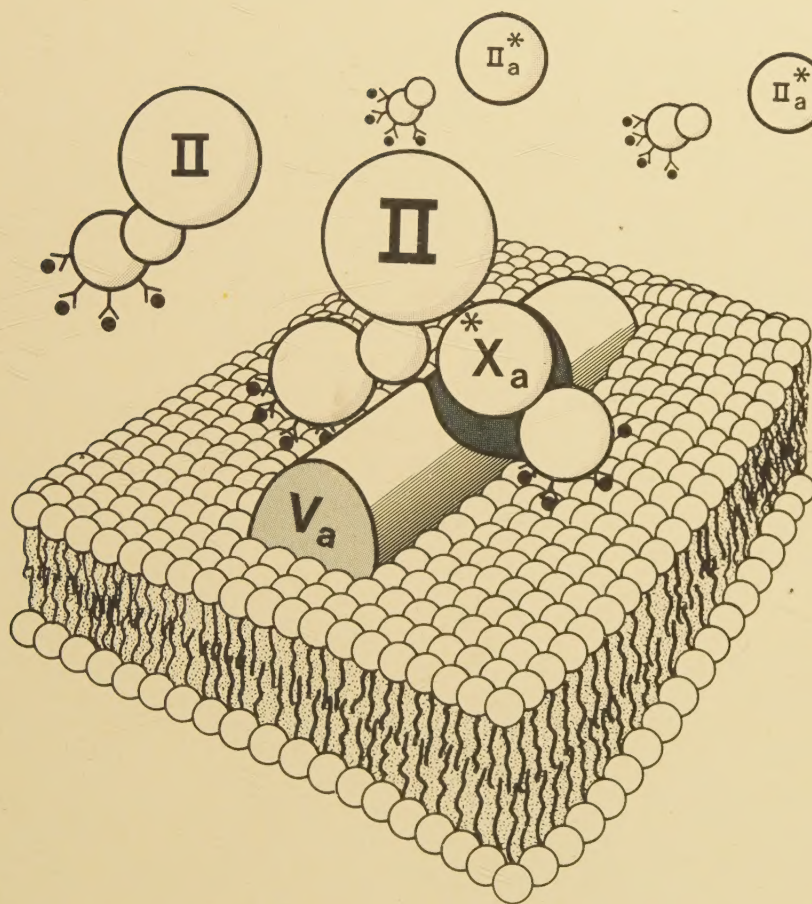


THE ACTIVATION OF PROTHROMBIN ON THE PLATELET SURFACE

BJÖRN DAHLBÄCK



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THE ACTIVATION OF PROTHROMBIN
ON THE PLATELET SURFACE

av

Björn Dahlbäck
leg läk, Mlm

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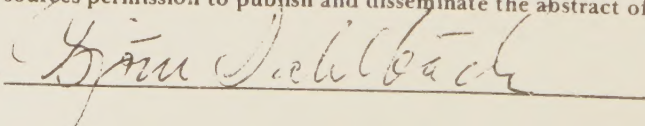
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Abstract Platelets are important not only for the primary hemostasis, but also for the blood coagulation by providing a catalytic surface for several of the reactions involved in the coagulation cascade. This thesis deals with the activation of prothrombin by factor X_a on the platelet surface. A small number of specific binding sites for factor X_a were exposed on the platelet surface after the release reaction. Factor X_a bound with high affinity and catalyzed the prothrombin activation effectively. The platelet factor X_a receptors consist of factor V_a and phospholipid. During its activation to thrombin the prothrombin molecule was found to interact with the factor V_a part as well as the phospholipid part of the factor X_a receptor. The kinetic parameters for the platelet bound activation(s) of prothrombin, acarboxyprothrombin (induced by the vitamin K antagonist dicoumarol), and prethrombin 1 (the prothrombin activation intermediate) were determined. The coefficient for proteolytic efficiency (k_{cat}/K_M) for prothrombin was 50 times that for acarboxyprothrombin indicating the importance of the vitamin K-dependent structures. The recently described vitamin K-dependent protein, protein C, was activated by thrombin formed in the platelet system. Activated protein C destroyed the factor X_a platelet receptors by proteolysis with inhibition of the prothrombin activation as a result. A simple purification procedure for human plasma factor V was devised. Like its bovine counterpart, human factor V was found to be a single chain molecule with an apparent molecular weight of 330,000. Well defined proteolytic cleavages occurred on incubation with a catalytic amount of thrombin.			
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THE ACTIVATION OF PROTHROMBIN ON THE PLATELET SURFACE

BJÖRN DAHLBÄCK

Malmö 1981

The present dissertation is a summary of the following communications referred to in the text by their Roman numerals:

- I. Binding of bovine coagulation factor X_a to platelets, (1978). Biochemistry, 17, 4938. In collaboration with J. Stenflo.
- II. The activation of prothrombin by platelet-bound factor X_a , (1980). Eur. J. Biochem., 104, 549. In collaboration with J. Stenflo.
- III. Inhibitory effect of activated protein C on activation of prothrombin by platelet-bound factor X_a , (1980). Eur. J. Biochem., 107, 331. In collaboration with J. Stenflo.
- IV. Human coagulation factor V purification and thrombin-catalyzed activation, (1980). J. Clin. Invest., 66, 583.



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INTRODUCTION

The first contribution to our knowledge of blood coagulation, i.e. how fluid blood transforms into a jelly-like mass when it leaves the body, was made by Malpighi in 1666, when he separated white fibrous strands from coagulated blood (1). In 1772 Hewson (2) localized fibrinogen, the precursor of these strands to plasma rather than to the blood corpuscles. The protein was purified considerably by Hammarsten in 1879 (3). In 1835 Buchanan showed that the formation of a fibrin clot was caused by an enzyme present in coagulated blood (4). This enzyme was later given the name thrombin by Schmidt (5). The conversion of fibrinogen to fibrin was studied in the nineteenth century and in 1905 Morawitz proposed the so-called "classical theory", according to which blood coagulation was thought to take place in two steps (6). In the first step tissue thromboplastin, which is a phospholipid-rich fraction obtained from homogenates of brain or lung, was believed to convert the then hypothetical substance prothrombin to thrombin. In a second step thrombin was assumed to convert soluble fibrinogen to fibrin strands.

Morawitz's classical theory soon proved to be too simple and many additional factors were found to be necessary for normal blood coagulation. In 1964 the results of extensive studies in many different laboratories using partially purified preparations of coagulation factors and plasmas from patients with congenital deficiencies of any of these factors led Macfarlane (7) and Davie and Ratnoff (8) independently to propose the cascade or waterfall theory as a possible model for the sequence of reactions involved in blood coagulation. Most clotting factors have since been isolated and chemically characterized (for reviews, see ref 9). This has enabled studies of the different reactions in *in vitro* systems with highly purified components. As a result of these studies the original cascade or waterfall model has been modified particularly regarding the role played by factors VIII and V, but the

general principles are still accepted (9,10). Fig. 1 illustrates a modified version of the coagulation cascade as it is understood today. Two pathways are traditionally distinguished, the so-called intrinsic and extrinsic pathways. The intrinsic system is initiated, in the test tube, when blood or

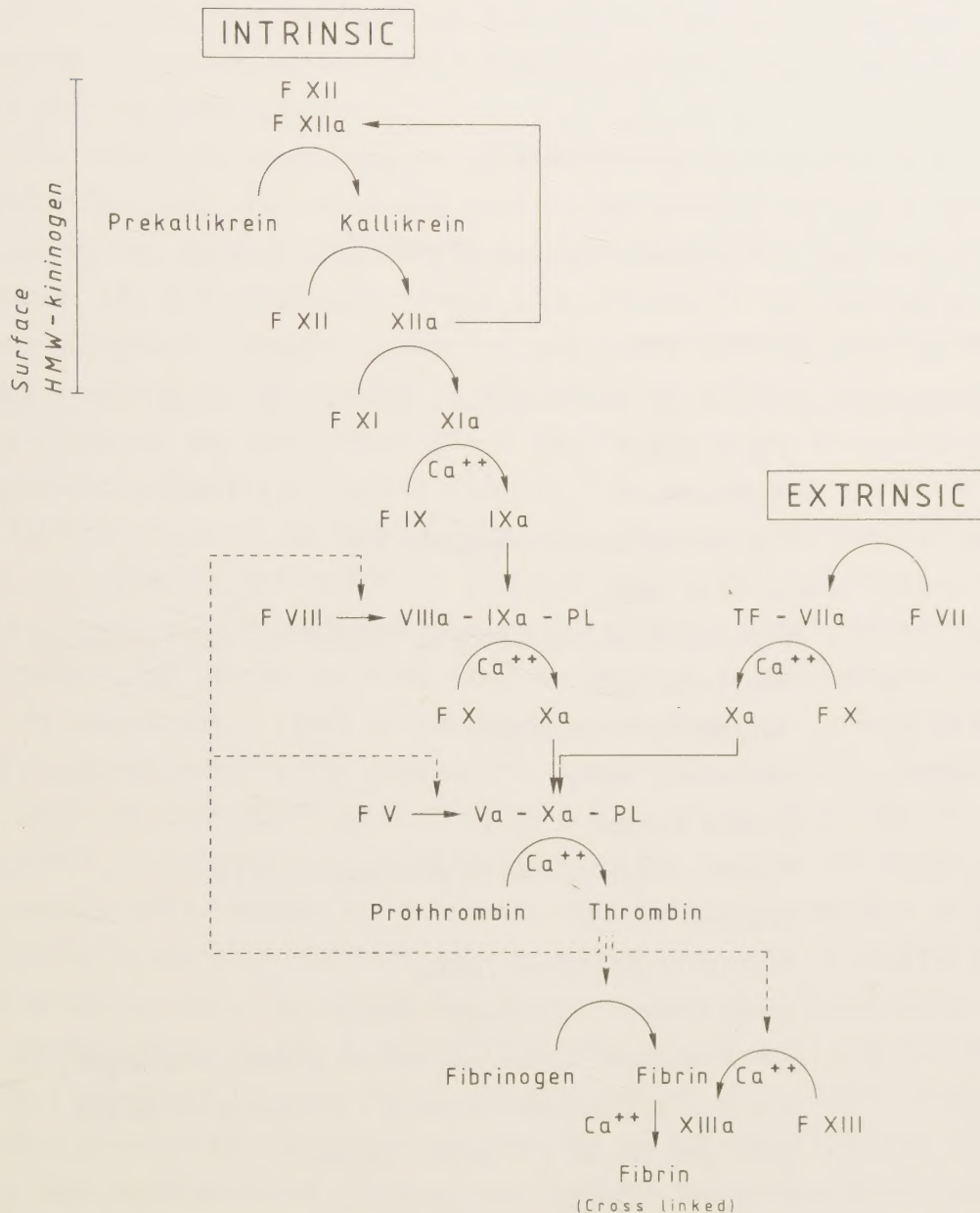


Fig. 1. Schematic model of the coagulation cascade. Subscript a denotes active enzyme. F stands for factor. HMW-kininogen, PL, and TF denote high molecular weight kininogen, phospholipid, and tissue factor, respectively. Modified from ref. 9,10,17.

plasma comes into contact with highly negatively charged surfaces, such as glass or kaolin, and all components involved are "intrinsically" present in blood. This is in contrast to the extrinsic system which is activated by exposure of blood or plasma to injured tissues which provide the tissue factor essential for this system. The common names of the coagulation factors and their Roman numbers are given in Table I.

Table I

Blood Coagulation Components

Roman Number	Common name
Factor I	Fibrinogen
Factor II	Prothrombin
Factor III	Tissue factor, thromboplastin
Factor IV	Calcium ions
Factor V	Proaccelerin, accelerator globulin, labile factor
Factor VII	Proconvertin
Factor VIII	Antihemophilic factor
Factor IX	Christmas factor
Factor X	Stuart factor
Factor XI	Plasma thromboplastin antecedent (PTA)
Factor XII	Hageman factor
Factor XIII	Fibrin stabilizing factor
-	Prekallikrein (Fletcher factor)
-	HMW-kininogen (high molecular weight kininogen, contact activation cofactor, Fitzgerald factor, Williams factor, Flaujec factor)
-	Protein C (autoprothrombin II _a)

Mechanisms of the coagulation cascade

The principal mechanism of the coagulation cascade is that of a sequential conversion of proenzyme (zymogen) to active enzyme leading to the formation of thrombin, which then transforms fibrinogen to fibrin. From one stage to the next the amount of proenzyme activated is increased resulting in a great amplification of a minute stimulus. The zymogens involved are proteins circulating in plasma in inactive form and in low concentrations. They are converted to active enzymes by limited proteolysis, i.e. cleavage of well defined peptide bonds (9,10).

The reactions take place on appropriate surfaces normally not exposed to circulating blood. The reactants bind to the surface and their local concentration by far exceeds that in circulating plasma. With the exception of thrombin, the active enzymes formed remain bound to the surface to participate in the subsequent zymogen activation. Negatively charged phospholipid membranes (exposed on the surface of disintegrated cells or aggregated platelets) serve as the catalytic surface for the reactions involving the vitamin K-dependent coagulation factors (factors VII, IX, X, and prothrombin) (9-14). The initial phase of the intrinsic system, the so-called contact activation phase, is *in vitro* activated and catalyzed by certain substances, such as glass or kaolin, but our knowledge of the cellular localization and biochemical composition of the surfaces involved *in vivo* is only meagre (10,15-17).

At almost every level of the cascade the catalytic effect of the enzyme is greatly enhanced by the presence of a specific accessory, nonenzymatic protein (i.e. factors VIII and V, high molecular weight kininogen or tissue factor) (9-11). The accessory protein binds to the surface and forms a complex with respectively enzyme and substrate. The reactants are thus arranged in a sterically favourable position and the activation rate is greatly catalyzed. The biological activity of the two important accessory components involved in the activation of factor IX and prothrombin, factor VIII and factor V, respectively, increases on exposure to a minute amount of thrombin, whereas higher thrombin concentrations have a destructive effect (10,11,18,19). This indicates that both positive and negative feedback mechanisms play important roles in the regulation of the rate of blood coagulation. Plasma protease inhibitors, like antithrombin III, are also important regulators of blood coagulation and they protect the circulation from potentially dangerous clotting enzymes (20). However, when an active clotting enzyme is part of a surface-bound macromolecular complex, it is protected from inhibition by antithrombin III (21,22).

The coagulation enzymes (with the exception of factor XIII) are serine

proteases, and the principal reaction sequence involved in the cleavage of peptide bonds in blood clotting is essentially identical with that described for pancreatic proteases (23). The amino acid sequences surrounding the active site serine of the coagulation enzymes are very homologous with trypsin, chymotrypsin, and elastase suggesting a common ancestor (10). There are, however, important differences between the coagulation and pancreatic serine proteases. The enzymes involved in clotting have a very high degree of substrate specificity and attack only one or two peptide bonds in their respective substrate. This, is in contrast to the digestion proteases from pancreas which, with little specificity, cleave peptide bonds involving the basic amino acids arginine or lysine (23). Furthermore, the coagulation enzymes are larger and more complicated molecules usually with two disulfide linked polypeptides - one containing the active site; the other, the surface binding site (10).

In summary the following basic principles are important in the function of blood coagulation: (1) All the zymogens and the accessory proteins involved are present at low concentration and in inactive form in circulating blood. (2) The coagulation cascade takes place on suitable surfaces normally not exposed to circulating blood. Active enzymes bound to these surfaces are protected from inactivation by plasma protease inhibitors. Owing to their surface binding, the local concentration of enzyme and substrate by far exceeds that in circulating plasma. (3) Specific accessory proteins arrange the reactants in a sterically favourable position leading to high activation rates. (4) Owing to a sequential arrangement of the reactions, a minute stimulus will be greatly amplified. (5) Specific positive and negative feed-back mechanisms regulate the activation rates. Furthermore, the coagulation process is regulated by the circulating plasma protease inhibitor, antithrombin III.

Initiation of coagulation

Important advances in our understanding of the contact activation reactions have recently been made (15-17,24). Today, it appears that four plasma proteins are involved: Hageman factor (factor XII), factor XI, prekallikrein, and high molecular weight kininogen. A variety of negatively charged materials, such as glass, kaolin, certain connective tissues or collagen preparations etc., activate and catalyze the system. Factor XII is the key enzyme for the contact activation reactions capable of initiating not only the intrinsic coagulation reactions, but also kinin formation and fibrinolysis. According to Heimark et al (17), in the presence of kaolin and high molecular weight kininogen, the single chain zymogen factor XII is as efficient in activating prekallikrein as is activated two chain factor XII (factor XII_a). Kallikrein is capable of converting single chain factor XII to two chain fac-

tor XII_a (15-17,24,25). Thus, the reciprocal activation of factor XII and prekallikrein works as a positive feed-back mechanism. Conversion of factor XI to XI_a, which leads to the activation of the coagulation pathway, however, is more effectively catalyzed by two-chain factor XII_a than by single chain factor XII. High molecular weight kininogen occupies a key position as a non-enzymatic cofactor for these surface-dependent reactions.

The current picture of the contact activation reactions is the result of *in vitro* experiments using nonphysiological stimulators, such as glass or kaolin. Thus, the role of the system in the initiation of coagulation under physiological conditions and the relative importance of the different reactions *in vivo* are still unknown. In this respect, it is important to point out that patients deficient in factor XII or prekallikrein have no hemostatic abnormality, whereas factor XI deficient patients show distinct signs of bleeding (26). This indicates that factor XII and prekallikrein, although important *in vitro* might not primarily be involved in the initiation of coagulation *in vivo*. The possible role of platelets in triggering off the intrinsic system via activation of factor XI is discussed later. The molecular mechanisms involved in the activation of the extrinsic system are also far from properly understood. Several enzymes, such as factor XII_a, kallikrein, factor XI_a, factor X_a, and thrombin have been shown to activate factor VII *in vitro*, but the physiological importance of these reactions is still obscure (10,27,28). The nonenzymatic cofactor in the extrinsic system is a lipoprotein complex called tissue factor and it is only exposed after tissue damage.

The important mechanisms involved in the activations of factors IX and X are not considered here, the reader being referred to recent reviews instead (10,28).

The prothrombinase complex

Macromolecular complexes are, as mentioned, formed in several stages of the coagulation cascade. The one characterized best is the prothrombinase complex, which consists of the enzyme factor X_a, the nonenzymatic accessory protein factor V_a (i.e. factor V activated by a small amount of thrombin), calcium ions, and phospholipid vesicles providing the catalytic surface. The current picture of the interactions involved in the prothrombinase complex is the result of extensive studies, in many different laboratories using well defined phospholipid mixtures and highly purified preparations of factor X_a, prothrombin, and factor V (10,11,28-36). Negatively charged phospholipids, particularly phosphatidylserine, properly mixed with neutral phospholipids, such as phosphatidylcholine and phosphatidylethanolamine, have been found to catalyze the reaction maximally. The catalyzing effect is not attributable

any particular phospholipid class, but to a negative charge of the phospholipid surface (11,37,38). The vitamin K-dependent structures in factor X_a and prothrombin (see below) mediate the calcium dependent binding of these proteins to the phospholipid surface (11,39). Factor V and V_a bind firmly to phospholipid vesicles in a process that is not calcium-dependent (40-44). Furthermore, factor X_a and prothrombin have an affinity for the nonenzymatic, accessory protein factor V, but only after it has been activated by a small amount of thrombin (45,46). As a result of these affinities a macromolecular complex is formed on the phospholipid surface leading to a rapid activation of prothrombin to thrombin by factor X_a .

Gamma-carboxyglutamic acid, the vitamin K-dependent structure

The discovery of the γ -carboxyglutamic acid residue first described in prothrombin (47-49) and later in factor IX and factor X (50-52) implied a major advance in our understanding of the interactions between the vitamin K-dependent coagulation proteins, calcium ions, and phospholipid surfaces. It has been demonstrated that the γ -carboxyglutamic acid residues in prothrombin are the result of a vitamin K-dependent postribosomal carboxylation of the first ten aminoterminal glutamic residues (11,39,53). Inhibition of this reaction by vitamin K-antagonists, such as dicoumarol, leads to the synthesis of an abnormal prothrombin molecule in which the glutamic acid residues are non-carboxylated (11,39,54-59). This abnormal form of prothrombin, which is referred to here as acarboxyprothrombin, does not bind Ca^{2+} and has no affinity to phospholipid membranes (59,60). This explains the potent anticoagulant activity of vitamin K-antagonists. As a result of the discovery of the γ -carboxyglutamic acid residue other vitamin K-dependent plasma proteins have been discovered and isolated to homogeneity, such as protein C (61,62), protein S (63-65), protein Z (66) and protein M (67). Protein C may be important since it might be a regulator of blood coagulation. After activation, protein C has distinct anticoagulant effects owing to the destruction of the thrombin activated forms of factors V and VIII, factor V_a and factor $VIII_a$, respectively (68-71,116). The physiological significance of the other recently described vitamin K-dependent plasma proteins is largely obscure. Vitamin K-dependent proteins have also been demonstrated in other tissues, such as bone and kidney (72).

Platelets in hemostasis and coagulation

Platelets are important in normal primary hemostasis (14,73,74). When a vessel wall is damaged, platelets adhere to subendothelial collagen with subsequent release of arachidonic acid from membrane phospholipids by the action of specific phospholipases (14,75-78). The arachidonic acid is conver-

ted to prostaglandin G_2 , H_2 and thromboxane A_2 , which together with liberated ADP induce adhesion and aggregation of adjacent platelets leading to the formation of a platelet plug (14). At the same time the platelets release their granula content including both high and low molecular weight components, such as fibrinogen, factor V, the heparin neutralizing platelet factor 4, serotonin, ADP etc (14,73,74). An increasing amount of experimental evidence produced in the last decade lends support to the concept of an intimate interrelationship between primary platelet plug formation and blood coagulation. According to a hypothesis, proposed in 1974 by Walsh (22), after adhesion to collagen platelets trigger off the intrinsic system by activating factor XI, and the subsequent clotting cascade occurs on the phospholipid surface of aggregated platelets, where the active coagulation factors are protected from inhibitors, such as antithrombin III. Further support for the hypothesis of such an intimate interrelationship between primary hemostasis and blood coagulation has been produced in recent experiments indicating that minute amounts of thrombin, well below the concentration that converts fibrinogen to fibrin, stimulate the platelet release reaction due to binding of thrombin to specific receptors on the platelet surface (79-86).

In 1977 Miletich et al demonstrated a specific platelet receptor for human factor X_a , a receptor, available only after the release reaction (87,88). The platelet bound factor X_a catalyzed the prothrombin activation 1000 times more efficiently than factor X_a bound to phospholipid vesicles indicating that platelets provided more than a pure phospholipid surface. It is now known that factor V_a is an important part of the factor X_a receptor, and that patients with severe congenital factor V deficiency lack the factor X_a receptor sites (89). The work by Miletich et al (87-89) produced the first direct evidence of an interaction between a vitamin K-dependent protein and a specific receptor site on the platelet surface, evidence that was important for the better understanding of blood coagulation.

PRESENT INVESTIGATION

The purpose of the present investigation was to elucidate the interactions between platelets and coagulation factors with special reference to factor X, prothrombin, factor V, and protein C. Mainly bovine materials were used because at least when this study started the bovine coagulation factors were easier to purify and were better chemically characterized than the corresponding human proteins. After an initial characterization of the binding of bovine factor X_a to bovine platelets (I) attention was directed to the structural properties of prothrombin necessary for its activation by platelet bound factor X_a (II). The role played by protein C in the platelet system were also investigated (III). A procedure for purifying human plasma factor V was devised (IV). The thrombin-catalyzed activation of human factor V was studied and compared with that reported for bovine factor V.

THE FACTOR X_a PLATELET RECEPTOR (I)^{*}

The addition of ^{125}I -labelled factor X_a to a mixture of purified prothrombin and washed platelets induces rapid thrombin generation after an initial lag phase (Fig. 2). During the lag phase the platelet release reaction is induced by a small amount of thrombin presumably formed on the phospholipid surface of disintegrated cells. The length of the lag phase seems to vary with the quality of the platelet preparation. Thus, the lag phase in experiments with platelets isolated by the gentle gel filtration technique is longer than that obtained with platelets isolated by differential centrifugation (90). When the release reaction is completed, factor X_a binds to the platelet surface and effectively catalyzes the activation of prothrombin. The platelet release reaction is a prerequisite, but the binding seems not to be influenced by the releasing agent. Thus, the same amount of factor X_a was bound to platelets treated with the calcium ionophore A 23187 as to platelets treated with a small amount of thrombin (I,87). The binding of factor X_a to the platelet surface is calcium-dependent but does not require the presence of prothrombin. Therefore, to avoid possible actions by thrombin and prothrombin activation fragments on the factor X_a binding it was characterized in a system devoid of prothrombin.

The binding of factor X_a to platelets has the same general characteristics as the binding of polypeptide hormones to cell surface receptors and seems to reflect a true receptor interaction. Factor X_a binds with high affinity (Table II) and the binding is specific, saturable, reversible and varies with the

^{*}The interaction between platelets and factor X_a has been studied in several laboratories (I,87,88,97). This chapter has, therefore, been written as a mini-review in an effort to give an overall picture of the subject. References have been given frequently in the text in an attempt to outline the contributions of the various laboratories.

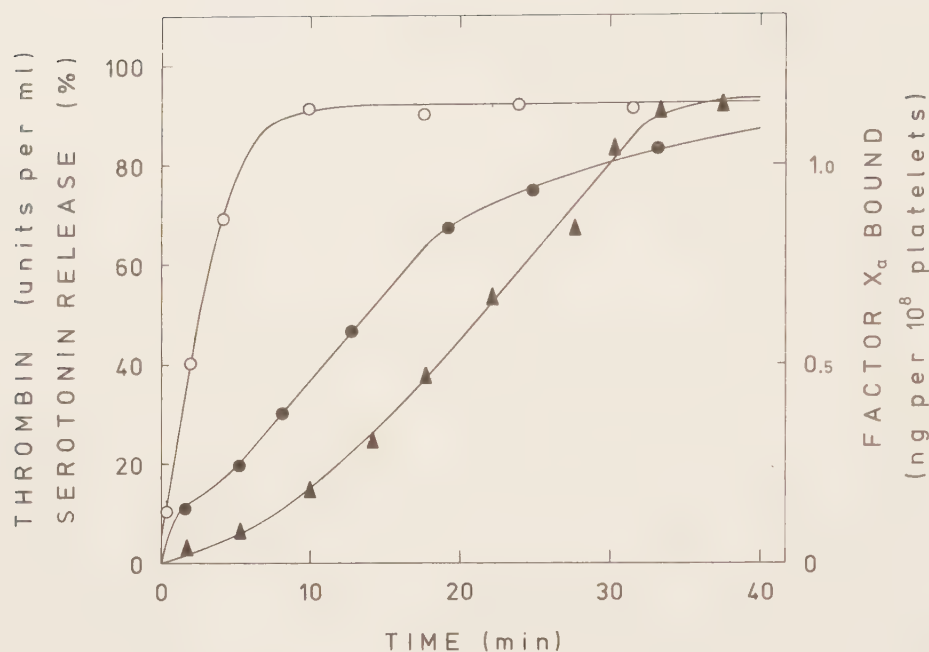


Fig. 2. Factor X_a binds to platelets after the release reaction and effectively catalyzes the activation of prothrombin. Platelets ($1 \times 10^8/\text{ml}$), preincubated with ^{14}C -serotonin, were mixed with prothrombin (0.1 mg/ml) in 50 ml Tris-HCl, 0.15 M NaCl, pH 7.4 containing bovine albumin (5 mg/ml), glucose (1 mg/ml) and 2.5 mM CaCl_2 . The reaction was started by the addition of ^{125}I factor X_a (20 ng/ml). (○), Serotonin release; (●), factor X_a binding; (▲), thrombin generation were followed as described (1). In this experiment factor X_a binding was not corrected for nonspecific binding.

biological response, i.e. with the rate of thrombin formation. The number of factor X_a binding sites (290-420 per platelet) is lower than the number of sites reported for the binding of polypeptide hormones to respective target cells (91) or the number of sites for thrombin on the platelet surface (80-84). The zymogen, factor X and related coagulation factors, such as factor IX (IX_a) and prothrombin, do not compete with factor X_a for the binding sites and this indicates the specificity of the receptor interaction (88). This is in line with a physiological importance of the factor X_a binding, i.e. in vivo presumably only a small fraction of factor X is converted to active enzyme and must bind to the platelet receptors in the presence of a large amount of other plasma proteins including factor IX, factor X, and prothrombin. Our results are in good agreement with the data reported for human factor X_a binding to

Table II

Factor X_a Binding To Platelets (I)

Association constant (M^{-1}) ^a	
Measured	5.2×10^9 (2.8×10^9 to 1.0×10^{10})
Calculated from $k+1/k-1$	2.7×10^9 (1.8×10^9 to 4.1×10^9)
Number of binding sites per platelet	290 - 420

^aThe average of six experiments on different days is given with the range in brackets.

human platelets (87,88). The number of binding sites for human factor X_a per platelet was approximately 400 and an affinity constant (K_A) of $3-4 \times 10^{10} M^{-1}$ was reported. The factor X_a -platelet interaction is obviously largely the same in each of the two species and the observation that bovine factor X_a binds equally well to human as to bovine platelets (unpublished work) indicates that the three dimensional structure of factor X_a has been extremely well preserved during evolution.

Factor V_a , part of the factor X_a receptor

The K_A for binding of bovine factor X_a to platelets (approximately $5 \times 10^9 M^{-1}$) is much higher than that reported by Nelsetuen et al (92) for the interaction between bovine factor X_a and phospholipid vesicles (approximately $4 \times 10^6 M^{-1}$) indicating that the platelet factor X_a binding sites do not consist of phospholipid alone. This concept is supported further by the fact that factor X_a associated with platelets catalyzes the prothrombin activation at least a 1000-fold more effectively than factor X_a bound to negatively charged phospholipid vesicles (87,88). The low number of binding sites per platelet and the high specificity are also observations in line with this concept, and the following data suggest that factor V, or more likely V_a , is part of the platelet factor X_a receptor. (1), The binding sites, like factor V_a from plasma, are destroyed by high concentrations of thrombin (87). (2),

Protein C_a is even more potent in this respect (III). (3), An antibody against factor V inhibits the binding of factor X_a to platelets (87). (4), Washed platelets contain a presumably unactivated form of factor V which appears on stimulation of platelets with thrombin, collagen or after freezing and thawing (93-96). (5), Miletich et al (89) showed that platelets from patients with congenital factor deficiency have a small number of factor X_a binding sites. The severity of the disorder varied more closely with the decrease in factor X_a binding than with the plasma factor V level. (6), Platelets have specific binding sites for factor V_a (95).

Factor V_a binding to platelets

Tracy et al (95) recently reported binding of bovine factor V and V_a to specific sites on the platelet surface available both before and after stimulation by thrombin. The binding was reversible and met the criteria for a true receptor interaction (91). Approximately 900 factor V_a molecules were bound per platelet with an association constant (K_A) of $3 \times 10^9 M^{-1}$. A second class of binding sites was also present and bound approximately 3500 factor V_a molecules per platelet with an association constant (K_A) of $3 \times 10^8 M^{-1}$. Unactivated factor V bound only to the lower affinity sites and could not displace the high affinity bound factor V_a . The K_A for the high affinity binding of factor V_a to platelets is many times that reported for the binding of factor V_a to phospholipid vesicles ($2 \times 10^6 M^{-1}$) (44). This indicates that the binding sites on the platelet surface for factor V_a do not consist of phospholipid alone. The nature of these sites is unknown. Of interest in this connection is that Miletich et al. (98) reported a bleeding disorder in a patient whose platelets contained a normal amount of factor V but a decreased number of factor V_a binding sites. Kane et al (99) demonstrated that both unstimulated and thrombin-activated human platelets can bind human factor X_a in the presence of added bovine factor V_a , but were unable to demonstrate any binding of factor X_a to platelets incubated with unactivated bovine factor V. This indicates that factor V_a rather than factor V constitutes the platelet factor X_a binding sites. Thrombin is the probable physiological activator of platelet factor V, but the observation that diisopropylphosphorfluoridate (DFP) inactivated factor X_a bound to platelets whether the release reaction was induced by thrombin or by the calcium ionophore A 23187 indicates that platelet factor V may, perhaps, be activated by a platelet protease during the release reaction. Such a platelet factor V activator has been suggested by Østerud et al (94).

Phospholipid, part of factor X_a receptor

The calcium dependence of factor X_a binding to the platelet surface sug-

gests that phospholipid is an important part of the receptor site. This concept is supported by an experiment, reported by Morita and Jackson^{*}, demonstrating that "headless" factor X_a (a modified factor X_a where the γ -carboxyglutamic acid containing region of the light chain had been removed by chymotryptic digestion) bound to the platelet surface with a much lower affinity than did intact factor X_a . As previously mentioned, the membranes of unstimulated platelets have an asymmetrical phospholipid distribution with negatively charged phospholipids (mainly phosphatidylserine) situated in the cytoplasmic layer (100). The observations that factor V_a binds to unstimulated platelets (95) to form factor X_a binding sites (99) is therefore intriguing and questions the importance of the interaction between the γ -carboxyglutamic acid residues in factor X_a and the negatively charged "procoagulant" phospholipids for platelet factor X_a binding. However, the differential centrifugation technique used by both groups to isolate platelets may cause changes in the very sensitive platelet surface. Indeed, Østerud et al (94) showed that unstimulated factor V deficient platelets isolated by gel filtration failed to bind factor V on incubation with normal plasma. But when platelets from the same patient were isolated by centrifugation they rapidly bound factor V from normal plasma. These findings stress how important it is to use an appropriate isolation technique in factor V (V_a) binding experiments.

In summary, platelets contain a presumably unactivated form of factor V, whose site of synthesis is unknown. Various stimuli induce the platelet release reaction and the activation of platelet factor V, which subsequently binds to specific receptors on the platelet surface. Factor V_a , from platelets or from plasma, bound as an integrated protein to the platelet phospholipid membrane, constitutes the factor X_a platelet receptor.

^{*}Reported by C.M. Jackson in a discussion at the International Workshop on Regulation of Coagulation, Oklahoma sept 4-8, 1979. Printed in Taylor, F.B. and Mann, K.G. (1980) The Regulation of Coagulation. Elsevier/North-Holland p 235.

ACTIVATION OF PROTHROMBIN BY FACTOR X_a BOUND TO PLATELETS (II)

Platelet bound factor X_a effectively catalyzes the activation of prothrombin. Miletich et al (87,88) reported that factor X_a is approximately 1000-fold more effective in activating prothrombin in the presence of platelets than in the presence of phospholipid. The same group later found that binding of factor X_a to platelet receptors accelerate prothrombin activation 300,000-fold, which was approximately 10-times more than the catalyzing effect of factor X_a and optimal concentrations of factor V_a and phospholipid. Also our results suggested factor X_a to be more effective in activating prothrombin when it is bound to platelets than when it is part of the prothrombinase complex (I). The value of these experiments, however, is limited by the fact that the factor V preparations available were not homogeneous, highly active, or well characterized. Later, Nesheim et al (101), purified bovine factor V to homogeneity and using that preparation as part of the prothrombinase complex observed a catalytic effect identical with that obtained in the platelet system (102). From kinetic data, they calculated that factor X_a and factor V_a in the prothrombinase complex interacted with a stoichiometric ratio of 1:1 (102). The K_A for the factor X_a interaction with factor V_a -phospholipid (also calculated from kinetics) was approximately $1.4 \times 10^{-9} M^{-1}$, which is similar to the K_A for factor X_a binding to bovine platelets (Table I) (I). In conclusion, when pure, native factor V_a is part of the prothrombinase complex it presumably serves as a viable model for the platelet system. However, the platelet system is more complex, as indicated by the observation of a limited number of the previously mentioned specific platelet factor V_a binding sites (95).

Pathway of prothrombin activation

The pathways of prothrombin activation by factor X_a in the presence of

factor V_a , phospholipid and Ca^{2+} have been extensively studied in several laboratories (11,29-36). A summary of the results obtained using bovine materials is given to facilitate understanding of the results observed in the platelet system. Factor X_a cleaves two peptide bonds in the prothrombin molecule (Fig. 3). Cleavage of the first bond results in formation of fragment 1-2 and the intermediate prethrombin 2. Prethrombin 2 has no thrombin activity and remains noncovalently associated with fragment 1-2 on the phospho-

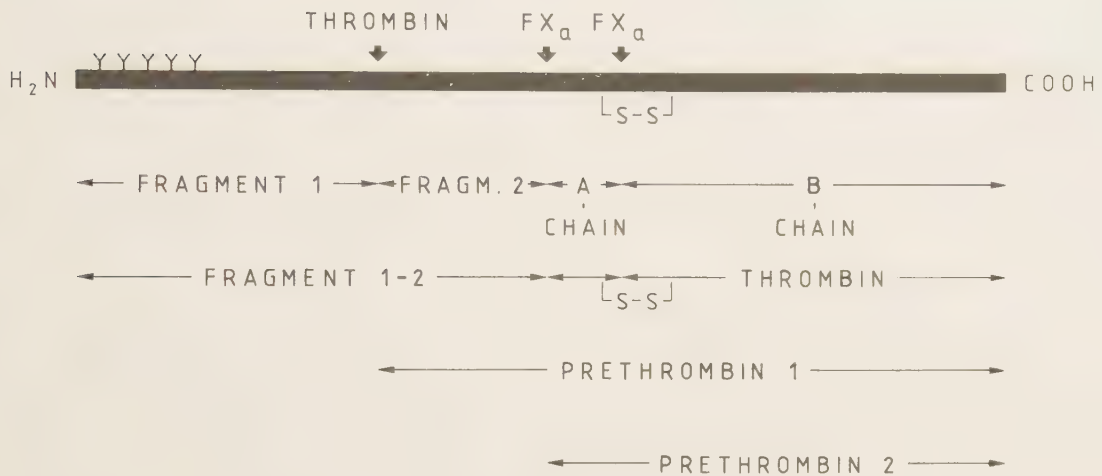


Fig. 3. Schematic model of the prothrombin molecule. The factor X_a - and thrombin-mediated cleavages are indicated by arrows. Nomenclature of fragments, intermediates, and products are given. The symbol (Y) denotes γ -carboxyglutamic acid residues.

lipid surface. Thus, the carboxyterminal "thrombin-half" of prothrombin does not leave the prothrombinase complex to diffuse into free solution until the second bond is cleaved by factor X_a . Thrombin is capable of cleaving one peptide bond in its own zymogen, prothrombin, to give rise to fragment 1 and prethrombin 1. The same peptide bond in fragment 1-2 is also susceptible to proteolysis by thrombin and its cleavage gives rise to fragment 1 and fragment 2. It has been proposed that the degradation of prothrombin to fragment 1 and prethrombin 1 is an important mechanism in the regulation of the rate of prothrombin activation (11) but it has not been possible to demonstrate its occurrence in vivo (103).

The ten γ -carboxyglutamic acid residues in prothrombin are located in the fragment 1 region (11). In the presence of Ca^{2+} they mediate the binding of prothrombin to negatively charged phospholipid (11). Isolated fragment 1 and prothrombin bind to the phospholipid vesicles with essentially the same characteristics, K_A approximately $0.5 - 1 \times 10^6 \text{M}^{-1}$ (104,92). Prethrombin 1 has no affinity to phospholipid vesicles, while its interaction with factor V_a seems to be indistinguishable from that of intact prothrombin (11). The factor V_a binding capacity is located in the fragment 2 region (11). The molecular mechanisms involved and the affinity of the interaction between factor V_a and prothrombin are still obscure. The unactivated form of factor V does not bind prothrombin or factor X_a , which is presumably of great physiological importance (45,46). Thus, the full biological activity of factor V is not exerted until a small amount of thrombin has modified the factor V molecule by limited proteolysis (105,106). Using a well defined system with purified components Nesheim et al (102) showed that the biological activity of bovine factor V increased approximately 400-fold on incubation with thrombin.

Interaction between platelets and prothrombin

Only few reports are available on prothrombin activation mechanisms and pathways in systems with platelets instead of the accessory components, factor V and phospholipid. Furthermore, little has been known about the direct interaction between platelets and prothrombin. In 1975 Tollefsen et al (107) reported that they could not demonstrate any binding of prothrombin to the surface of intact platelets. They used a Millipore filtration assay that included a tenfold dilution of the suspension before filtration and a washing procedure with 10 volumes of buffer. Weak interactions would probably not be detected after such a disturbance in the equilibrium. Using a more refined oil centrifugation technique we tried to demonstrate binding of prothrombin to platelets both before and after induction of the release reaction but without success (I). The difficulties in showing prothrombin binding to platelets are probably due to the fact that K_A is relatively low and the number of binding sites is small. Direct binding techniques, thus proved unable to characterize the prothrombin-platelet interaction, which was therefore studied by determining the structural requirements of the prothrombin molecule for it to be activated by platelet bound factor X_a (II). Activation rates of prothrombin, acarboxyprothrombin (prothrombin lacking γ -carboxyglutamic acid residues), prethrombin 1, and prethrombin 2 were compared to estimate the relative importance of the prothrombin interactions with phospholipid and factor V_a in the platelet system. Acarboxyprothrombin and prethrombin 1 have no

affinity for phospholipid. Prethrombin 2 is devoid of both fragment 1 and fragment 2 regions and therefore has no affinity for phospholipid or for factor V_a .

The prothrombin activation fragments, intermediates, and products were found to be the same in the presence of factor X_a and platelets as in the presence of factor X_a , phospholipid, and factor V_a . This indicates that the two systems catalyze the activation of prothrombin by similar mechanisms and pathways and, thus, that the results of the two systems can be used for comparisons.

To find out whether the vitamin K-dependent structures in prothrombin are necessary for rapid prothrombin activation by platelet bound factor X_a the activation of purified acarboxyprothrombin and that of normal prothrombin were compared (Fig. 4). In the control with normal prothrombin, platelets rapidly release their serotonin content on addition of factor X_a . After

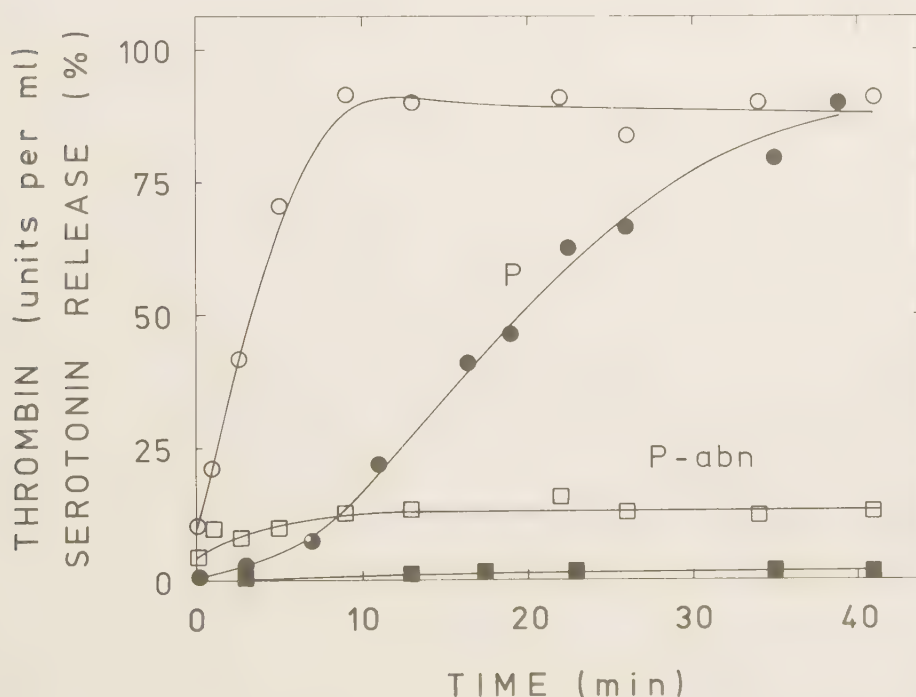


Fig. 4. Activation of prothrombin by factor X_a in the presence of platelets; comparison between normal and acarboxyprothrombin. Factor X_a (8 ng/ml) was added to parallel platelet (1×10^8 /ml) reaction mixtures with normal or acarboxyprothrombin (0.15 mg/ml). (○) Serotonin release and (●) thrombin generation in incubation mixture with normal prothrombin (P); (□), serotonin release and (■), thrombin generation during incubation with acarboxyprothrombin (P-abn).

a short lag phase, when factor X_a binds to the platelet receptors, thrombin was rapidly generated. In the reaction mixture containing acarboxyprothrombin no serotonin was released and no thrombin was generated during the first 30-40 minutes. However, when the release reaction was induced by the calcium ionophore A 23187 a very slow thrombin generation started immediately and thereby indicated that acarboxyprothrombin interacts with platelet bound factor X_a presumably because of the affinity of the fragment 2 part of the molecule for platelet factor V_a . The difference in activation rates of prothrombin and acarboxyprothrombin lends support to the concept that the vitamin K-dependent structures in prothrombin are very important for the interaction between prothrombin and the platelet surface.

To assess the importance of the factor V_a - prothrombin interaction in the platelet system and compare it with the phospholipid binding, thrombin generation was followed in parallel platelet reaction mixtures with equimolar concentrations of prothrombin, prethrombin 1 and prethrombin 2 (Fig. 5). To assure that factor X_a receptors were available the platelet release reaction was induced before the addition of factor X_a . Prothrombin was converted to thrombin approximately ten times as fast as prethrombin 2 and three times as fast as prethrombin 1 indicating that both phospholipid and factor V_a binding parts of prothrombin, i.e. fragment 1 and fragment 2 respectively, are necessary for a rapid activation. The addition of fragment 2 to reaction mixtures containing prethrombin 2 increased the activation rate. In equimolar concentrations of the two the activation rate was largely the same as that obtained in an incubation mixture containing the same molar concentration of prethrombin 1. The catalyzing effect of fragment 2 on the activation of prethrombin 2 is due to a strong noncovalent association of the two which resulted in a complex functionally identical with prethrombin 1 (108). Thus, the fragment 2 - prethrombin 2 complex, in contrast to prethrombin 2, has an affinity for platelet factor V_a which explains the observed increased activation rate.

The activation rates of prothrombin, prethrombin 1, and acarboxyprothrombin were functions of the amount of factor X_a bound to the platelets and indicated that the reactions took place on the platelet surface. Maximal rates were noted when the factor X_a receptors were saturated. The kinetic parameters for the three substrates were measured and the results are given in Table III. The K_M of 0.6 μM prothrombin is very similar to the K_M recently reported for the prothrombinase complex (102,109), and the turnover number (k_{cat}) 10 mol of thrombin/sec/mol factor X_a bound is in agreement with both that reported for the human factor X_a - platelet system (88) and those reported for factor X_a consisting part of the prothrombinase complex (102,109).

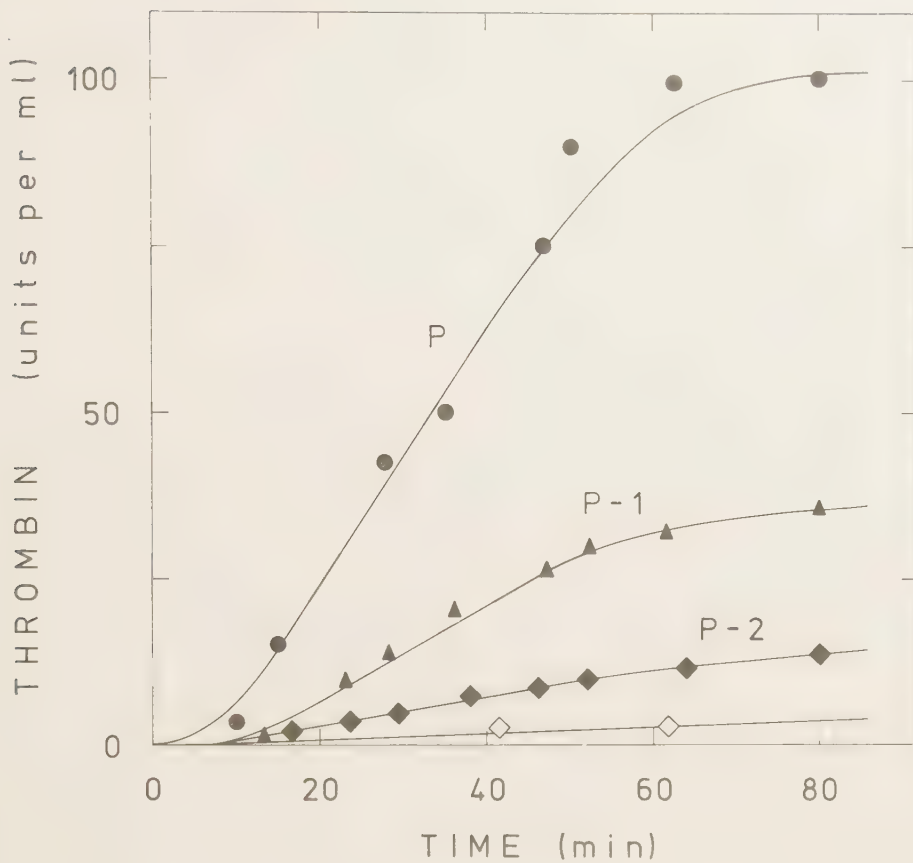


Fig. 5. Comparison between thrombin generation in parallel platelet reaction mixtures with prothrombin, prethrombin 1, or prethrombin 2. The substrate concentrations were equal ($3 \mu\text{M}$) and the release reactions were induced with the calciumionophore A 23187 ($1 \mu\text{M}$) before the addition of factor X_a (200 ng/ml), (●), prothrombin (P); (▲), prethrombin 1 (P-1); (◆),^aprethrombin 2 (P-2); (◇), parallel reaction mixture with prethrombin 2 but without platelets.

The coefficient for proteolytic efficiency ($k_{\text{cat}}/K_{\text{M}}$) found with the use of prothrombin as substrate was 20- and 50-times higher than those obtained with prethrombin 1 and acarboxyprothrombin, respectively.

Conclusion

Available data appear to warrant the conclusion that both platelet phospholipid and factor V_a are involved in, and important for, the interaction between prothrombin and the platelet surface. Factor X_a and prothrombin form an enzyme-substrate complex on a limited number of sites consisting of factor V_a and platelet phospholipid (Fig. 6). Susceptible bonds in prothrombin

Table III

Kinetic Parameters for Prothrombin Activation in the Presence of Platelets (II)

	$K_M(\mu M)$	$k_{cat}(s^{-1})$	$k_{cat}/K_M(s^{-1}\mu M^{-1})$
Prothrombin ^a	0.6(0.4-0.8)	10	16.7
Prethrombin 1 ^b	3.6(2.9-4.4)	3.0	0.83
Acarboxyprothrombin ^c	3.6(3.6-3.6)	1.3	0.36

Average values are given with the range in brackets.

^afive determinations, ^bthree determinations ^ctwo determinations

are exposed to proteolysis by factor X_a with rapid thrombin generation as a result. Factor X_a binds to the platelet receptor with high affinity ensuring that the binding sites are saturated even at a very low factor X_a concentration. Thus, one per cent activation of plasma factor X (equal to approximately 100 ng per ml) would be sufficient to saturate the platelet factor X_a receptors. Prothrombin presumably interacts with a much lower affinity than does factor X_a . Assuming a dissociation constant (K_D) of 1.5-3 μM , half of the platelet receptor sites for prothrombin would be occupied at a prothrombin concentration equal to that of plasma (approximately 1.5-3 μM). Furthermore, if one assumes that the rate of association for prothrombin is similar to that determined for factor X_a binding to the platelet receptors (I) the half-life of the complex should be less than a second. This would explain the difficulties in demonstrating direct binding of prothrombin to platelets even with the rapid oil centrifugation technique used in factor X_a binding experiments.

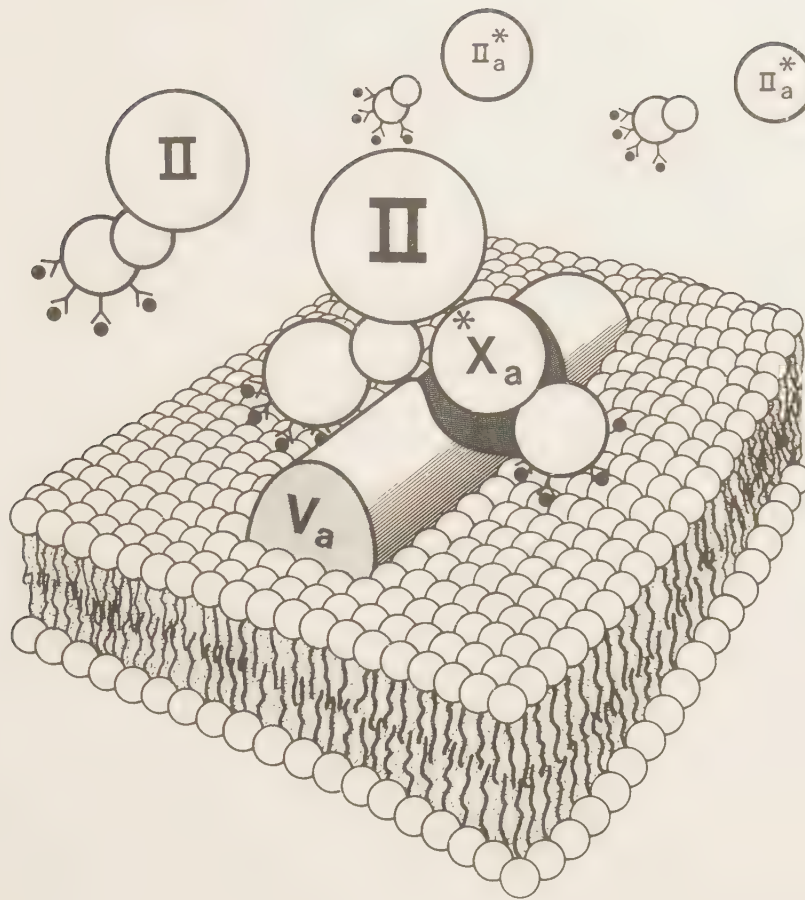


Fig. 6. Schematic model for the interaction between factor-X_a (X_a), factor V_a (V_a), and prothrombin (II) on the platelet surface. The symbol (Λ^a) denotes γ-carboxyglutamic acid residues and (●) Ca²⁺. (II_a^{*}) denotes thrombin formed and (*) active site serine.

PROTEIN C, A POTENTIALLY IMPORTANT REGULATOR OF BLOOD COAGULATION (III)

Protein C is a vitamin K-dependent protein normally occurring in relatively low concentration (approximately 5 mg per liter) in plasma. It was originally purified from bovine plasma in 1976 (61). Recently, the corresponding human protein was isolated and found to be functionally and structurally closely related to its bovine counterpart (62). Human and bovine protein C are zymogens of serine proteases and are activated both by thrombin (62,68) and the factor X activator from Russell's viper venom (110). During activation, an activation peptide 14 amino acid residues long, is released from the aminoterminal part of the heavy chain. The active site serine is located in the heavy chain, whereas the vitamin K-dependent γ -carboxyglutamic acid residues are situated in the aminoterminal part of the light chain. The approximate molecular weights of the heavy and light chains are 41,000 and 21,000, respectively. The amino acid sequences of both heavy and light chains have recently been determined (111,112). The amino acid sequence of the aminoterminal part of the light chain and the sequence around the active site serine are highly homologous to corresponding regions of the other vitamin K-dependent proteins. Comparison of the complete sequences of the light chains in protein C and factor X has shown that they are closely related, whereas after the last γ -carboxyglutamic acid residue in the light chain of protein C (position 35) the sequence homology with prothrombin is no longer striking (111).

Activated protein C (protein C_a) is identical with autoproteins II-A, which 20 years ago Seegers and his coworkers (113) described as an inhibitor of blood coagulation. According to their hypothesis, autoproteins II-A is derived from the prothrombin molecule. In 1972, Marcianik questioned this hypothesis and thought that autoproteins II-A was a separate protein (114). This objection proved justified in 1976 when purified protein C was shown to

be the zymogen of autoprothrombin II-A (113). Seegers' group described the anticoagulant activity of autoprothrombin II-A as a competitive inhibition of factor X_a (115). This assumption has been proved to be incorrect, and today the anticoagulant effect of protein C_a is thought to be due to a proteolytic degradation of factor V and factor VIII (68-71). Recent data indicate that protein C_a selectively degrades the activated forms of factor V and factor VIII (71,116). The rate of inhibition of factors V_a and $VIII_a$ by protein C_a is greatly enhanced by the presence of phospholipid and Ca^{2+} , which indicates that enzyme-substrate complexes are formed on the phospholipid surface. In summary, protein C after activation by thrombin may be an important regulator of the blood coagulation cascade by the selective destruction of factors V_a and $VIII_a$ bound to phospholipid membranes. In this respect it is important that antithrombin III does not inhibit protein C_a even in the presence of heparin (68). Recently, Marlar and Griffin (117) reported that protein C_a is inhibited by a plasma protein distinct from previously described protease inhibitors. Their observation that four patients with combined factor V/VIII deficiency lacked the protein C_a inhibitor lent further support to the concept that protein C_a is important for the regulation of factors V_a and $VIII_a$ in vivo.

Protein C_a destroys the factor X_a receptor

As previously discussed, the factor X_a platelet receptor consists of factor V_a bound to the platelet phospholipid membrane. It was therefore of interest to investigate whether protein C_a can destroy the platelet factor X_a binding sites and function as an inhibitor of the prothrombin activation in the platelet system. An inhibitory effect on the rate of prothrombin activation was observed when protein C_a was included in a platelet - factor X_a - prothrombin reaction mixture. Relatively high concentrations of protein C_a (1-10 $\mu\text{g/ml}$) were necessary to obtain a significant inhibition. We found that the inhibitory effect of protein C_a is due to proteolytic destruction of the factor X_a platelet receptors. Thus, when thrombin-stimulated platelets were preincubated for various periods with protein C_a before the addition of factor X_a and prothrombin, a time-dependent parallel decrease in factor X_a binding and rate of prothrombin activation was found. This effect was only observed when platelet mixtures were used in which the release reaction had been induced before the addition of protein C_a . The zymogen, protein C, or protein C_a inactivated with DFP had no inhibitory effect on the platelet system whether the release reaction had been induced or not. Furthermore, we could not demonstrate direct binding to the platelet surface of the activated or the zymogenic form of protein C. Results in good agreement with those we

obtained have also been reported independently by Comp and Esmon (118). Available data indicate that protein C_a exerts its inhibitory effect by proteolytic degradation of the factor V_a part of the factor X_a receptor.

Thrombin may be the activator of protein C in vivo. However, in vitro this reaction is not particularly effective and according to Kisiel et al (110), protein C isolated from plasma and serum are indistinguishable and protein C is not activated during coagulation of plasma. The physiological conditions during blood coagulation in vivo are different from those in the test tube and their experiments do not rule out a local protein C activation in vivo. In this connection it is noteworthy that we observed activation of protein C presumably by thrombin formed during prothrombin activation in a platelet - factor X_a - prothrombin reaction mixture. The rate of protein C activation was relatively low, which may suggest that a cofactor was missing.

In conclusion, the specific destruction of the phospholipid bound thrombin activated forms of the nonenzymatic, accessory components factor V and factor VIII lend support to the concept that protein C_a is an important regulator of blood coagulation in vivo. It can be speculated that protein C is "the" most important local regulator, whereas the physiological purpose of antithrombin III (which, as previously mentioned, is incapable of inhibiting phospholipid bound clotting enzymes) may be to protect the rest of the circulation from potentially dangerous clotting enzymes.

PURIFICATION AND THROMBIN-CATALYZED ACTIVATION OF HUMAN FACTOR V (IV)

Factor V, also called labile factor, accelerator globulin, or proaccelerin, was first identified by Owren in 1947 (119) as an important component of the coagulation system. Its extreme susceptibility to proteolysis is the main reason why it is so difficult to isolate it in pure and unmodified form. It was not until 1979 that Esmon (105) and Nesheim et al (101) independently demonstrated the successful purification of undegraded bovine factor V. Both groups stressed the importance of rapid handling and the addition of protease inhibitors throughout the purification procedure. The successful isolation of native bovine factor V shed valuable light on its function, structural properties, and pathway of activation (44,95,101,102,105,106,120). It is a single chain glycoprotein with a molecular weight of 330,000 as determined by ultracentrifugation (101). As previously mentioned, its activity as an accessory component of the prothrombinase complex increases dramatically after proteolysis by a catalytic amount of thrombin (102,105).

Human factor V is even more labile than its bovine counterpart, and the few purification procedures on record have not yielded undegraded, homogeneous factor V (18,121,122). However, the information gained by the isolation of bovine factor V improved the prospect of a successful purification of human factor V. The last part of this thesis is concerned with the isolation and thrombin-catalyzed activation of human factor V.

The purification procedure devised for undegraded human factor V is illustrated in Fig. 7. Some important principles are stressed. The starting material should be either fresh or freshly frozen plasma and an abundance of protease inhibitors must be added at every step. Of further importance are quick handling, a low temperature, and the presence of Ca^{2+} to stabilize the factor V molecule.

PURIFICATION OF HUMAN FACTOR V

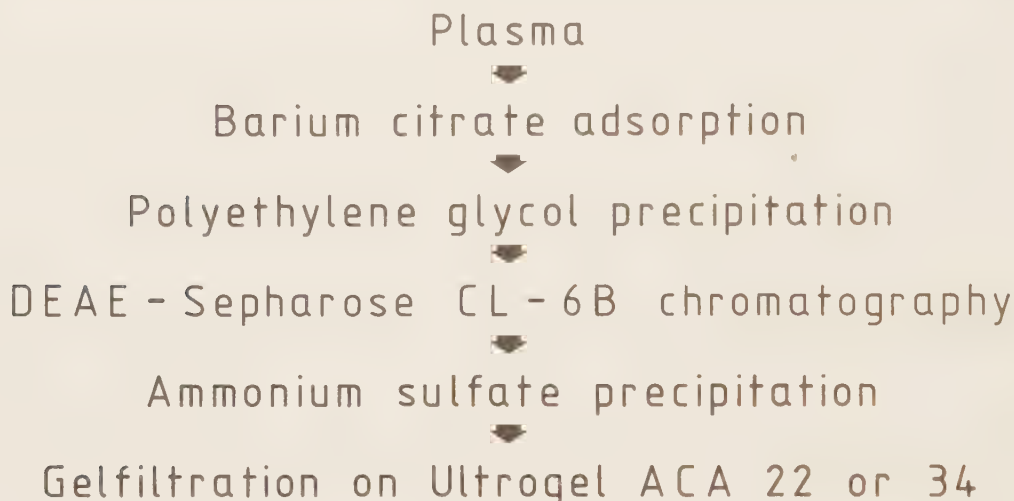


Fig. 7. Steps in purification of single chain human factor V.

In the present investigation dialysis and concentrations by ultrafiltration were avoided. The first step was barium citrate adsorption of the vitamin K-dependent proteins to avoid formation of thrombin and protein C_a during subsequent steps. This was followed by fractionated polyethylene glycol precipitation which rapidly reduced both the sample volume and the amount of proteins and resulted in a four- to five-fold purification. The dissolved precipitate was immediately applied to a DEAE-Sepharose CL-6B chromatography. Factor V bound very firmly to the column and was eluted at a high ionic strength. The eluted factor V was concentrated by ammonium sulfate precipitation and then subjected to a gel filtration chromatography using an acrylamide - agarose based resin (Ultrogel ACA 22 or 34). After dialysis against 50 % glycerol/buffer the pure factor V was stored for at least four to six months without demonstrable loss of activity or degradation into lower molecular weight species. The isolated factor V was purified approximately 1500-fold relative to the starting material but the recovery was low. The appearance of partially proteolysed factor V, which was eluted late during the

DEAE-Sepharose chromatography, indicated that the amount of protease inhibitors used in the original work (IV) was too small. A much better yield (25-30 %) was obtained by increasing the protease inhibitor concentrations during all steps. The purified protein obtained by this modified procedure was mainly undegraded single chain factor V, demonstrated in Fig. 8 (Suzuki, Dahlbäck, and Stenflo to be published). Human and bovine factor V comigrated on reduced SDS-polyacrylamide gels indicating identical molecular weights, i.e. 330,000.

Human Factor V

— 330 K

R

Fig. 8. Human factor V; pattern on 5 % polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate. The right sample was reduced, but not the left. Approximately 20 μ g was applied to each gel.

The addition of a catalytic amount of thrombin to a sample with single chain human factor V resulted in an increase in factor V activity with a concomitant proteolysis of the high molecular weight form (Fig. 9). The extreme susceptibility of human factor V to thrombin is illustrated by the rapid disappearance of the 330,000 molecular weight component even at the low initial temperature and despite the presence of the protease inhibitor benzamidine. The pattern obtained was compared with that of bovine factor V, and the



Fig. 9. Activation of single chain human factor V by thrombin. Human factor V (0.6 mg/ml) was incubated with human thrombin (1 μ g/ml) in 50 % glycerol, 25 mM Tris-HCl, 50 mM NH_4Cl , pH 7.5, 5 mM CaCl_2 , 5 mM benzamidin pH 7.5. The reaction mixture was initially kept on an ice bath and after 15 minutes the temperature was shifted to 37°C. At various intervals aliquots were removed, reduced and subjected to SDS-gradient (5-15 %) polyacrylamide slab gel electrophoresis. The different components are indicated. The position of component C_1 (see text) is given. This experiment was performed in collaboration with Dr Koji Suzuki and Dr Johan Stenflo.

nomenclature of Nesheim and Mann (106) was adopted. A proposed model for the thrombin-catalyzed activation of human factor V is given in Fig. 10 together with that reported for bovine factor V (106). Human component C_1 is not visible in gels stained with Coomassie Brilliant Blue, but can be identified using a ^{125}I -labelled factor V (Suzuki, Dahlbäck and Stenflo to be published). Although the patterns, on SDS-polyacrylamide gels, of bovine and human factor V activations are distinct, the proposed activation models are very similar. The major difference between the two species seems to be the order of bond cleavages which explains why component C_1 is not seen in the bovine system whereas component C (note: distinct from component C_1) is apparently not an intermediate during activation of human factor V. Detailed studies over the

pathways of activation of human factor V and the effects of protein C_a in this system are in progress in collaboration with Dr Koji Suzuki and Dr Johan Stenflo.

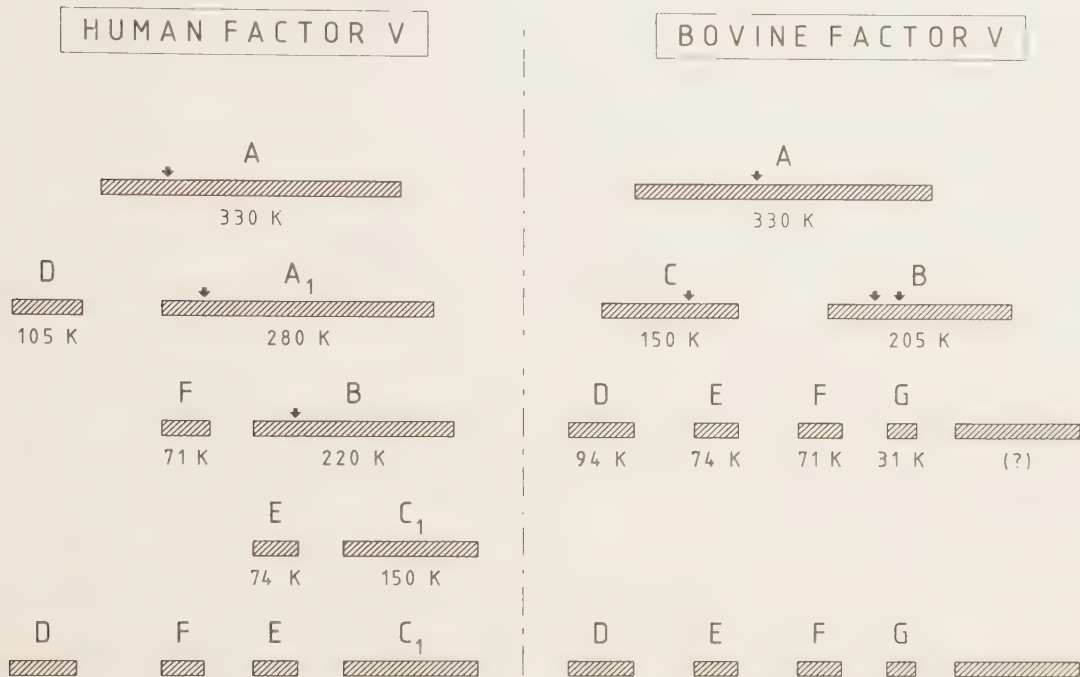


Fig. 10. Proposed schematic model for thrombin-catalyzed activation of human factor V. The approximate molecular weights are given, K stands for thousand. The experimental data forming the basis of this model will be published later (Suzuki, Dahlbäck, and Stenflo). The activation model presented by Nesheim and Mann is given for comparisons (106). Arrows indicate peptide bond cleavages.

SUMMARY

The surface of aggregated platelets catalyzes several of the sequentially arranged zymogen activations involved in the blood coagulation cascade. This thesis concerns the activation of prothrombin by factor X_a in the presence of platelets.

Factor X_a binds with high affinity to a small number of specific receptor sites exposed on the platelet surface after the release reaction. The binding sites consist of an activated form of factor V bound as an integrated protein in the platelet phospholipid membrane. Factor X_a bound the receptor sites catalyzes the prothrombin activation several 100,000-fold more effectively than factor X_a in free solution. Direct binding of prothrombin to the platelet surface could not be demonstrated presumably because of low affinity in combination with the smallness of the number of binding sites. Therefore, the platelet-prothrombin interaction was studied by determination of the structural requirements on the prothrombin molecule for it to be activated by platelet bound factor X_a . The vitamin K-dependent γ -carboxyglutamic acid residues in prothrombin were found to be necessary for rapid activation, an observation indicating the importance of the interaction with the phospholipid membrane. The fact that the fragment 2 part of prothrombin, which interacts with factor V_a , was also important demonstrates that prothrombin has an affinity not only for platelet phospholipid, but also for the factor V_a part of the factor X_a receptor. From the experimental data it is concluded that factor X_a and prothrombin form an enzyme-substrate complex on a limited number of sites on the platelet surface. The binding sites consist of factor V_a and suitable phospholipid. Prothrombin binds with low affinity exposing susceptible bonds for high affinity bound factor X_a . The

reaction sequence described is presumably important for a local activation of prothrombin in vivo.

Activated protein C was found to destroy the factor V_a part of the factor X_a receptor by proteolysis. This reaction may be the physiologically important mechanism regulating the rate of prothrombin activation on the platelet surface. In this connection it is important to note that platelet bound factor X_a is protected from inhibition by antithrombin III (88) and that antithrombin III is incapable of inhibiting activated protein C (68).

The relative importance and the structural relation of platelet and plasma factor V are still obscure. The purification procedure devised for homogeneous, undegraded factor V can be an important instrument in the future elucidation of this important question.

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Binding of Bovine Coagulation Factor X_a to Platelets[†]

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ABSTRACT: The binding of highly purified bovine coagulation factor X_a to washed bovine platelets was studied. ¹²⁵I-labeled factor X_a underwent binding to a platelet receptor that became accessible only after induction of the platelet release reaction by thrombin or by the calcium ionophore A 23187. The zymogen factor X did not bind to platelets. The factor X_a binding was saturable, reversible, and correlated with the rate of thrombin formation. The number of factor X_a binding sites per platelet was 290–420 and the apparent association constant was estimated to be 2.8×10^9 to $1.0 \times 10^{10} \text{ M}^{-1}$. Diisopropyl fluorophosphate–factor X_a bound to the same platelet receptor

as factor X_a indicating that limited proteolysis of a receptor protein was not required for binding. The rate of factor X_a binding was rapid (2.1×10^6 to $2.9 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) and similar to that previously found for the rate of binding of polypeptide hormones to their receptors. Displacement of factor X_a from the platelet receptor by diisopropyl fluorophosphate–factor X_a effectively blocked thrombin formation. Low concentrations of factor X_a catalyze prothrombin activation more effectively in the presence of platelets than in the presence of phospholipid and factor V.

Platelets have a central role in hemostasis (Weiss, 1975; Gordon, 1976). Subsequent to vascular injury, platelets adhere

to subendothelial collagen and aggregate to each other forming a so called hemostatic plug. This process is accompanied by a release reaction, i.e., exocytosis of platelet granules with release of both high and low molecular weight components such as fibrinogen, Ca²⁺, serotonin, and adenosine disphosphate. The aggregated platelets appear to provide a catalytic surface for localized activation of the plasma clotting factors. This

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process leads to fibrin formation and consolidation of the hemostatic plug.

The last step in the blood coagulation cascade (Davie & Fujikawa, 1975) is the conversion of prothrombin to thrombin by factor X_a . The rate of activation of prothrombin by factor X_a is, in the presence of calcium ions, greatly accelerated by phospholipid and factor V (Suttie & Jackson, 1977). Both factor X_a and prothrombin bind to factor V (Suttie & Jackson, 1977; Freeman et al., 1977) and to phospholipid vesicles (Suttie & Jackson, 1977; Nelsestuen & Lim, 1977; Nelsestuen & Broderius, 1977; Lim et al., 1977; Dombrose et al., 1978). The binding to phospholipid requires presence of calcium ions (Suttie & Jackson, 1977; Dombrose et al., 1978). In vitro platelet lipid extracts stimulate blood clotting and platelet lipoproteins are even more active in this respect (Marcus et al., 1966). Information on the interaction of the vitamin K-dependent proteins with platelets or platelet subcellular structures has, however, been lacking. It was therefore an important step forward when Miletich et al. (1977) demonstrated that human factor X_a bound to platelets that had undergone the release reaction and catalyzed thrombin formation, whereas the zymogen, factor X, did not bind to the platelets (Miletich et al., 1977). The factor X_a receptor appeared to be a protein and had certain properties in common with factor V (Miletich et al., 1977).

The interaction between prothrombin and phospholipid vesicles has recently been studied in detail (Suttie & Jackson, 1977; Lim et al., 1977; Nelsestuen, 1976; Nelsestuen et al., 1976). Very little is known, however, about the physiologically important interaction between prothrombin and platelets. Tollefsen et al. (1975) reported that they could not demonstrate binding of prothrombin to platelets. Using an oil centrifugation technique that ensures rapid separation of platelet bound and free prothrombin (Miletich et al., 1977; Feinberg et al., 1974; Martin et al., 1976), we have also been unable to demonstrate binding of prothrombin (1 ng/mL to 0.25 mg/mL) to platelets both prior to and following induction of release. This may be due to a very rapid rate of dissociation of prothrombin from a platelet receptor. Other possibilities are that a prothrombin receptor must be proteolytically modified by factor X_a before actual binding of prothrombin can occur or that the factor X_a molecule itself is part of the prothrombin receptor. Before studying interaction between prothrombin and platelets we therefore wanted to characterize the factor X_a binding to bovine platelets in some detail. Bovine platelets were used in these experiments since bovine plasma is available in large amounts and since the vitamin K dependent proteins from bovine plasma are more easily purified and far better chemically characterized than the corresponding human proteins. This paper reports on the characterization of the factor X_a binding to bovine platelets.

Materials and Methods

Factor VII and X deficient plasma, Russell's viper venom, the taipan snake venom (*Oxyuranus scutellatus scutellatus*), Folch fraction III, rabbit brain cephalin, diisopropyl fluorophosphate (Dip-F¹), phenylmethanesulfonyl fluoride $\text{PhCH}_2\text{SO}_2\text{F}$, and bovine serum albumin (Cohn fraction V) were from Sigma Chemical Co, St. Louis, Mo.; apiezon oil was from J.B. Biddle Co and di-*n*-butyl phthalate from the British Drug House, Poole, England. [¹⁴C]Hydroxytryptamine binoxalate (44 μCi per μmol) was from New England Nuclear,

Dreieichenhain, West Germany, and Na^{125}I , carrier free, from the Radiochemical Center, Amersham, England. The calcium ionophore A 23 187 (Lilly) was a kind gift from Dr Ingemar Lundkvist, University of Lund, Lund, Sweden. Millipore filters (RAW 025-00), 1.2- μm pore diameter, were from Millipore AB, Gothenburg, Sweden. Bovine fibrinogen (90% clottable) was from Miles Laboratories, Inc., Kankakee, Ill.

Bovine prothrombin was purified as described previously (Stenflo, 1976). It was dialyzed against 0.02 M Tris-HCl, 0.15 M NaCl, pH 7.35, and stored at a protein concentration of 6.7 mg per mL at -70°C . Prothrombin was quantified using an $E_{1\text{cm}}^{1\%}$ at 280 nm of 14.6 (Stenflo, 1972). Bovine thrombin was prepared by activation of bovine prothrombin with the taipan snake venom and purified by QAE-Sephadex chromatography (Owen & Jackson, 1973). It had a fibrinogen clotting activity of 620 NIH units per mg of protein. Highly purified bovine factor V was a generous gift from Dr. Peter Esnouf, Radcliff Infirmary, Oxford, England. The specific activity of factor V was given to be 80 units per mL per absorbance unit at 280 nm when the factor V activity of bovine plasma was defined to be 1 unit per mL. Incubation with thrombin did not further increase the factor V activity. A phospholipid suspension was prepared from Folch fraction III, dissolved to 50 mg per mL in chloroform:methanol (2:1), and evaporated to dryness. Tris-HCl, 0.02 M, 0.15 M NaCl, pH 7.35, was added to a final phospholipid concentration of 20 mg per mL buffer and the suspension sonicated in a Branson sonication bath under N_2 until transparent (40–50 min).

Factor X. Bovine factor X was purified to homogeneity as described previously (Stenflo, 1976). Activation of factor X was carried out with the factor X activator from Russell's viper venom (Schiffman et al., 1969) as described by Jesty & Nemerson (1976). The active enzyme was isolated by DEAE-Sephadex Chromatography, concentrated by Amicon ultrafiltration using the UM 10 filter, dialyzed against 0.05 M Tris-HCl, 0.1 M NaCl, 50% (v/v) glycerol, pH 7.5, and stored at -20°C . Factor X was quantified spectrophotometrically using an $E_{1\text{cm}}^{1\%}$ at 280 nm of 12.4 (Jackson, 1972).

The factor X_a preparations were initially homogeneous on agarose gel electrophoresis in 0.075 M barbital buffer, 2 mM EDTA, pH 8.6 (Johansson, 1972). After a few weeks at -20°C a slight decrease in factor X_a activity was noticed and a narrow protein band more cathodal than factor X_a appeared. This cathodal protein (30% or less of the total protein), presumably a degradation product of the active protein, had no factor X_a activity. It gave a faint immunoprecipitate on crossed immunoelectrophoresis (Ganrot, 1972) with a monospecific antiserum against factor X (Stenflo, 1976) but did not bind to platelets. Two preparations of factor X_a were used. No difference in binding to platelets was observed between these two preparations.

Inactivation of factor X_a by DFP was performed essentially as described by Fujikawa et al. (1972). The factor X_a (0.26 mg/mL) was incubated with 5×10^{-3} M DFP in 50 mM Tris-HCl, 0.15 M NaCl, pH 7.35, for 2 h at 37°C . The pH did not fall below 7.0 during the incubation. Remaining active factor X_a , less than 0.1 %, was removed by filtration through a column (0.9 $\times$ 5 cm) of Sepharose 4B with covalently linked soybean trypsin inhibitor (Cuatrecasas, 1970). The eluate had no factor X_a activity. The DFP inactivated factor X_a was electrophoretically (Johansson, 1972) and immunochemically (Ganrot, 1972) indistinguishable from factor X_a . It was stored at $+4^\circ\text{C}$. Factor X_a (0.25 mg/mL) was also inactivated with $\text{PhCH}_2\text{SO}_2\text{F}$ by incubation with 2×10^{-3} M $\text{PhCH}_2\text{SO}_2\text{F}$ in 20 mM Tris-HCl, 0.15 M NaCl, pH 7.35, overnight at 22°C . After this treatment less than 0.1% factor X_a activity remained.

¹ Abbreviations used: Dip-F, diisopropyl fluorophosphate; $\text{PhCH}_2\text{SO}_2\text{F}$, phenylmethanesulfonyl fluoride.

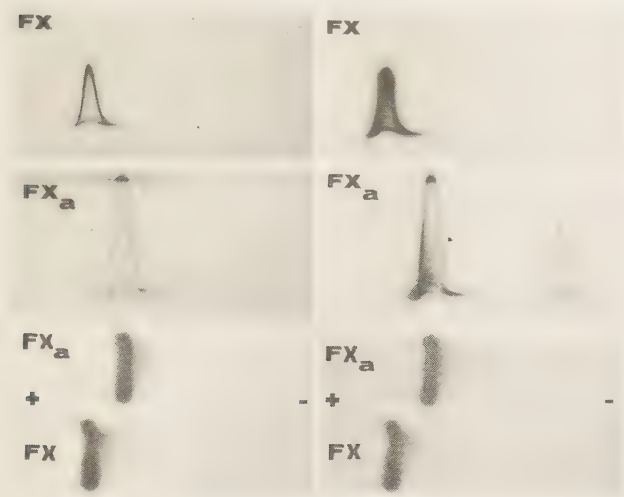


FIGURE 1: Patterns obtained by antigen-antibody crossed immunoelectrophoresis of labeled factor X and factor X_a in the presence of carrier. Protein staining to the left and autoradiography to the right. Below is shown the agarose gel electrophoretic patterns of unlabeled factor X and factor X_a. The agarose gel electrophoresis was run in 0.075 M barbital buffer, pH 8.6, containing 2 mM EDTA.

This preparation was used only in displacement experiments.

Clotting Assays. All assays were done in a Fibrometer coagulation timer (BBL). The method of Fenton & Fasco (1974) was used to determine thrombin activity. The assay was standardized with a thrombin standard, lot no. B-3, kindly supplied by Dr. D. L. Aronsen of the Department of Health, Education and Welfare, Bethesda, Md. Factor X_a activity was assayed according to Backman et al. (1958) using Cephalin without Russell's viper venom and factor VII and X deficient test plasma.

To study generation of thrombin in the presence of platelets, 0.8 mL of prothrombin, 0.17 mg/mL in 20 mM Tris-HCl, 0.15 M NaCl, pH 7.35, containing 5 mg per mL of bovine serum albumin and 1 mg per mL of glucose was mixed with 0.1 mL of 25 mM CaCl₂ and 0.1 mL of platelets, 10⁹ per mL, and incubated with factor X_a at 22 °C. Thrombin activity was measured in the fibrometer. In some experiments phospholipid and/or factor V was used instead of platelets.

Iodination Procedure. Factor X, factor X_a, Dip-F-factor X_a, and prothrombin were iodinated with sodium [¹²⁵I]iodide by a lactoperoxidase method essentially as described by Thorell & Johansson (1971). Free iodide was removed by gel filtration on a column of Sephadex G-25 (PD 10 Pharmacia Fine Chemicals), equilibrated with 20 mM Tris-HCl, 0.15 M NaCl, pH 7.35. The protein containing fractions were pooled and stored in 100-μL aliquots at -70 °C after the addition of bovine serum albumin to 5 mg/mL. The specific radioactivities of ¹²⁵I-labeled factors X and X_a, Dip-F factor X_a, and prothrombin were 800–1000 Ci/mmol and the mole ratios of iodide to factors X and X_a, DIP-factor X_a, and prothrombin were from 0.36 to 0.5. Iodinated factor X and prothrombin were immunochemically identical with their unlabeled counterparts. The characterization of labeled factor X_a is described under Results.

Isolation and ¹⁴C Labeling of Platelets. Platelets were isolated from bovine blood according to the method of Tollefsen et al. (1974) but with citrate as an anticoagulant instead of EDTA. Nine parts of bovine blood, drawn by jugular vein puncture, were collected in a plastic bottle containing 1 part of 2.85% trisodium citrate solution. The washed platelets were

suspended in concentration of approximately 10⁸ per mL and 2 μL (0.2 μCi) of [¹⁴C]hydroxytryptamine binoxalate (44 μCi per μmol) was added per mL. The suspension was incubated for 30 min at 37 °C, washed twice by centrifugation at 1300g for 10 min, and resuspended in 20 mM Tris-HCl, 0.15 M NaCl, pH 7.35, containing bovine serum albumin (5 mg/mL), glucose (1 mg/mL), and 2.5 mM CaCl₂ to a final concentration of 1 × 10⁸ per mL. The platelets were counted in a Technicon platelet autounter. The isolated platelets had a normal shape when inspected in the phase contrast microscope.

The Release Reaction and Binding Measurements. The release reaction was induced in 2–10 mL of the platelet suspension either by adding 1 unit of bovine thrombin per mL of reaction mixture or the calcium ionophore, A 23 187 (Lilly), to a final concentration of 1 μM. Immediately after the thrombin or Ca ionophore addition, the platelet suspension was carefully mixed. Binding experiments were made at ambient temperature, 22–24 °C, in (11 × 55 mm) polystyrene tubes. The binding of proteins to platelets and platelet release was measured by the oil centrifugation technique (Miletich et al., 1977; Feinberg et al., 1974; Martin et al., 1976). The reaction mixture (0.05–0.1 mL) was carefully layered on 0.1 mL of an oil mixture (1 part apiezon, 9 parts *n*-butyl phthalate) in 0.4-mL plastic centrifuge tubes. After centrifugation in a Beckman microfuge for 1 min, the bottoms of the tubes with the platelet pellets were cut off and the radioactivity was measured in a γ counter. The counting error was less than 2%. Platelet serotonin release was measured by determining [¹⁴C]serotonin in aliquots of supernatants from parallel incubations from which iodinated proteins were omitted. Radioactivity was measured in Packard Instagel in a liquid scintillation counter. One hundred percent release was estimated by counting ¹⁴C in an aliquot of the platelet suspension without prior centrifugation.

When inspected under the phase contrast microscope, the thrombin and ionophore treated platelets had typical appearance for platelets that have undergone the release reaction. There was no sign of aggregation during the first 30 min but after that time both microscopic and macroscopic aggregation occurred. The aggregation tendency was greatly enhanced when the platelet suspensions were stirred. There was no evidence of platelet lysis using 1 μM Ca ionophore concentration, whereas 2 μM or higher ionophore concentrations induced rapid platelet lysis.

In some dissociation experiments platelet bound factor X_a was separated from free factor X_a by Millipore filtration. The filters were washed with ice-cold buffer, 20 mM Tris-HCl, 0.15 M NaCl, pH 7.35, 5 mg/mL bovine serum albumin, 1 mg/mL glucose, and 2.5 mM CaCl₂, and radioactivity was measured in a γ counter.

Results

Characterization of Iodinated Factor X_a. Factor X_a had the same electrophoretic mobility on agarose gel electrophoresis prior to and after labeling. Crossed immunoelectrophoresis of labeled factor X_a with monospecific rabbit anti-factor X antiserum and unlabeled factor X_a as carrier showed that factor X_a is less readily recognized by the antiserum than the zymogen. Autoradiography revealed that radioactivity was confined to the immunoprecipitates (Figure 1). For both factor X and X_a, 85% of the radioactivity was recovered in the corresponding protein band on the agarose gel when the immunoprecipitates were cut out and the radioactivity was counted. The cathodal degradation product present in some factor X_a preparations did not bind to platelets. No correction was made for the presence of this protein in the binding studies. [¹²⁵I]-

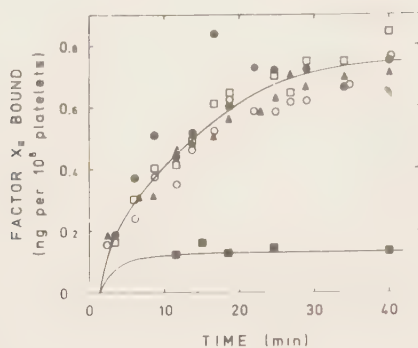


FIGURE 2: Comparison of platelet binding of factor X_a of different specific radioactivities. 125 I-labeled factor X_a was diluted with unlabeled factor X_a . Parallel platelet suspensions previously treated with 1 unit of thrombin per mL were incubated with 20 ng per mL of the different 125 I-labeled factor X_a solutions and the binding to platelets was measured as a function of time. (●) 22 000 cpm per ng of factor X_a (100% labeled factor X_a); (□) 16 500 cpm per ng of factor X_a ; (▲) 11 000 cpm per ng of factor X_a ; (○) 5500 cpm per ng of factor X_a ; (■) nonspecific binding.

Dip-F factor X_a gave the same precipitation pattern as 125 I-labeled factor X_a on crossed immunoelectrophoresis.

The rate of thrombin production induced by 125 I-labeled factor X_a in the presence of platelets varied between 20 and 100% of that of unlabeled factor X_a . The factor X_a activity of 125 I-labeled factor X_a also varied between 20 and 100% of that of unlabeled factor X_a . It was therefore necessary to exclude the possibility of different binding behavior between labeled and unlabeled protein. Factor X_a solutions with different specific activities were made from two labeled factor X_a preparations with low biological activity by mixing iodinated and unlabeled factor X_a . When binding to released platelets was studied as a function of time using these solutions, no significant differences were found (Figure 2). In another experiment when bound factor X_a was measured as a function of total factor X_a concentration, the preparations with different specific activities showed identical binding. More than a fourfold dilution with unlabeled factor X_a was, however, not compatible with precise radioactivity measurements in the platelet pellets. These experiments indicate that despite the slight variation in biological activity of 125 I-labeled factor X_a the binding to released platelets of the labeled protein is not differed from that of the unlabeled protein as seen by the identical binding curves.

Binding of 125 I-Labeled Factor X_a to Platelets. Binding of factor X_a to platelets, in which the release reaction had been induced with 1 U thrombin per mL, was measured as a function of factor X_a concentration. More than ten experiments were performed with nearly identical results. One experiment is shown in Figure 3. Nonspecific binding was estimated in parallel platelet- 125 I-labeled factor X_a incubations in the presence of a 100-fold excess of unlabeled factor X_a and used for further corrections. The corrected binding was saturable at levels of 2–3 ng of 125 I-factor X_a per 1×10^8 platelets. This corresponds to 290–420 binding sites per platelet according to Scatchard plots. The specific binding required that platelet release had occurred and in most experiments equilibrium was reached after about 15 min, whereas nonspecific binding was rapid and nonsaturable. The amount of factor X_a bound was independent of releasing agent used (thrombin or Ca ionophore). The nonspecific binding to released platelets corresponded to the total binding to unreleased platelets. The degree of binding of factor X_a to the platelets appeared to correlate to the degree of platelet serotonin release as observed in experiments where calcium ionophore concentrations below 1

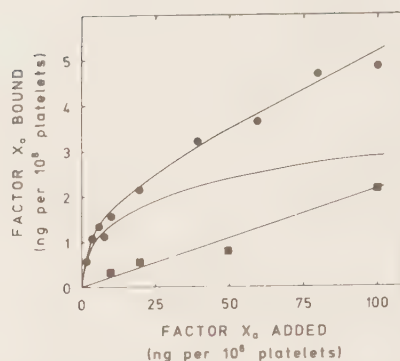


FIGURE 3: Binding of 125 I-labeled factor X_a to platelets as a function of the 125 I-labeled factor X_a concentration. Before incubation, platelet release was induced with 1 unit of thrombin per mL. Binding was measured after 30-min incubation. Nonspecific binding of 125 I-labeled factor X_a was measured in parallel reaction mixtures in the presence of 100-fold excess of unlabeled factor X_a . The specific binding of 125 I-labeled factor X_a was obtained by subtraction of the nonspecific binding from the total 125 I-labeled factor X_a bound. (●) Binding of factor X_a ; (■) nonspecific factor X_a binding; (—) specific factor X_a binding.

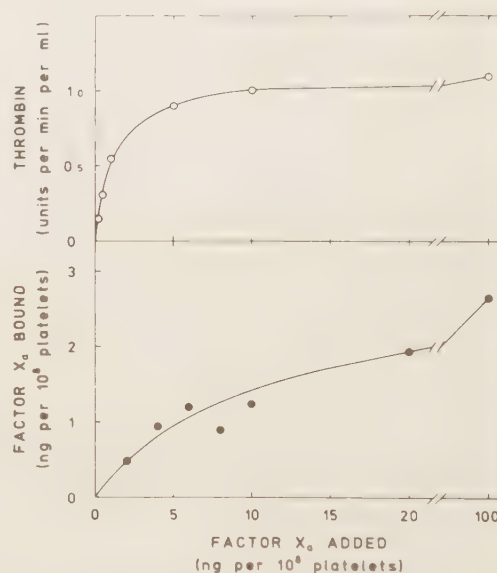


FIGURE 4: Correlation between the rate of thrombin formation and factor X_a binding. Above, rates of thrombin formation in parallel reaction mixtures containing platelets (1×10^8 per mL), prothrombin (1 mg per mL), Ca^{2+} (2.5 mM), and increasing concentrations of factor X_a . Below, binding of 125 I-labeled factor X_a to platelets as a function of 125 I-labeled factor X_a concentration (no prothrombin present). The platelets were treated with 1 unit of thrombin per mL before the incubation was started. The binding was measured after 30-min incubation and the binding data were corrected for nonspecific binding. (○) Rate of thrombin formation; (●) specific factor X_a binding.

μ M were used. The specific binding required calcium in the incubation mixture. No specific binding of the zymogen, factor X , was observed whether platelet release had been induced or not. The binding data, plotted as bound vs. total factor X_a and a dose-response curve showing the rate of thrombin formation as a function of factor X_a concentration, are shown in Figure 4. The dose-response curve was made with prothrombin concentration of 1.0 mg per mL to ensure that the prothrombin concentration was not rate limiting. The correlation between the factor X_a binding curve and the rate of thrombin formation was good, although the maximal rate of thrombin formation was reached at approximately 50% of the saturating factor X_a concentration. The rate of thrombin formation at each factor X_a concentration was measured from the linear part of the

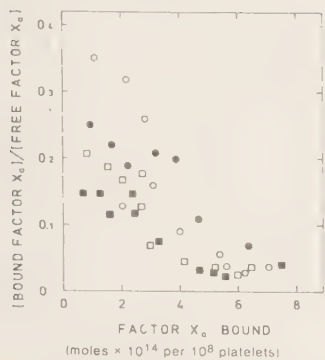


FIGURE 5: Scatchard plots of specific ¹²⁵I-labeled factor X_a binding data from four different experiments on different days.

TABLE I: Rate and Equilibrium Constants for Binding of Factor X_a to Platelets.

rate constants ^a	
$k_{+1}(\text{assoc}) \text{ (M}^{-1} \text{ s}^{-1}\text{)}$	2.4×10^6 (2.1×10^6 to 2.9×10^6)
$k_{-1}(\text{dissoc}) \text{ (s}^{-1}\text{)}^b$	9×10^{-4} (7×10^{-4} to 1.2×10^{-4})
assoc constant $\text{(M}^{-1}\text{)}^a$	
k_A^c	5.2×10^9 (2.8×10^9 to 1.0×10^{10})
calcd K_A^d	2.7×10^9 (1.8×10^9 to 4.1×10^9)

^a The average from three to six determinations on different days is given with the range in parentheses. ^b Determined by filtration assay (see the text). ^c From Scatchard plots. ^d Calculated from K_{+1}/K_{-1} .

curve obtained after complete platelet release had occurred. Prothrombin was not present in the reaction mixtures used to measure factor X_a binding.

The binding data from four experiments plotted according to Scatchard are shown in Figure 5. The interexperimental variation seen in the figure is largely due to variation inherent in the platelet counting procedure. The binding data are, within experimental error, compatible with the existence of one class of binding sites. The intercept on the abscissa in several experiments indicated a limiting value of 4.8×10^{-14} to 7.0×10^{-14} moles of factor X_a bound per 10^8 platelets (290–420 molecules per platelet) and from the intercept on the y axis an apparent association constant of 2.8×10^9 to $1.0 \times 10^{10} \text{ M}^{-1}$ was calculated (Table I). From the dose–response curves the concentrations of factor X_a required to give half-maximal thrombin formation rates were found to be 1–1.5 ng/mL. This would give an estimated association constant of $3\text{--}4 \times 10^{10} \text{ M}^{-1}$. A possible explanation for this somewhat higher value may have been that there was an influence of prothrombin on the factor X_a binding.

Rate of Binding and Dissociation of Factor X_a–Receptor Complex. Factor X_a binds only to platelets that have undergone the release reaction. Release is generally complete within 2 to 3 min on incubation of 1×10^8 platelets, with 1 U thrombin per mL. When iodinated factor X_a was added with the thrombin, maximum binding was not obtained until after 10 to 20 min. Identical results were obtained with calcium ionophore released platelets. Serotonin is released from dense granules in the platelet whereas the factor X_a receptor might have been associated with some other type of granule. To rule out the possibility that the rate of factor X_a binding depends

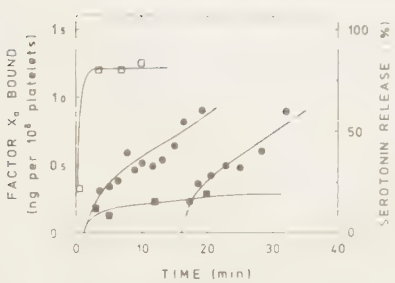


FIGURE 6: Rate of binding of ¹²⁵I-labeled factor X_a to isolated platelets. One milliliter suspension (10^8 per mL) was incubated with 20 ng of ¹²⁵I-labeled factor X_a. The incubations were started 75 s and 16 min after induction of the release reactions with 1 unit of thrombin. The binding data were not corrected for nonspecific binding. [¹⁴C]Serotonin release was measured in a parallel platelet suspension where the labeled factor X_a was omitted. A platelet suspension that had not been treated with thrombin was also incubated with 20 ng per mL of ¹²⁵I-labeled factor X_a and the binding was followed as a function of time. (●) Factor X_a binding to platelets after the release reaction; (■) binding to platelets that had not undergone the release reaction; (□) serotonin release.

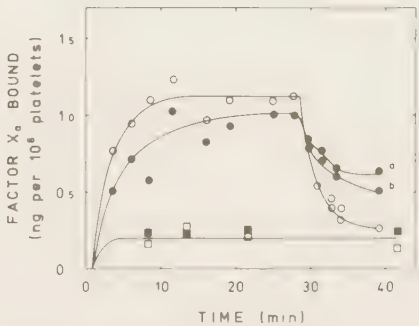


FIGURE 7: Binding of ¹²⁵I-labeled factor X_a and [¹²⁵I]Dip-F-factor X_a to platelets as a function of time. The platelet suspension was treated with 1 unit of thrombin per mL, before incubation with 20 ng per mL of ¹²⁵I-labeled factor X_a or [¹²⁵I]Dip-F-factor X_a. Binding was measured in aliquots of 50 μ L as described in Materials and Methods. After 28 min of incubation, a 100-fold molar excess of unlabeled factor X_a (b) or unlabeled Dip-F-factor X_a (a) was added to portions of the reaction mixtures and the displacement of the labeled proteins measured. (●) Binding of factor X_a; (○) binding of Dip-F-factor X_a; (■) nonspecific binding of factor X_a; (□) nonspecific binding of Dip-F-factor X_a.

on another platelet release reaction that was slower than serotonin release, the rate of factor X_a binding was measured with the centrifugation technique when ¹²⁵I-labeled factor X_a was added both immediately after the induction of serotonin release and approximately 15 min thereafter. From Figure 6 it is obvious that the rate of binding was identical in both cases.

When the rate of formation of the factor X_a receptor complex was studied, it was noticed that nonspecific binding was always maximal at the first measurement (2–8 min). All binding data were corrected for nonspecific binding as described. Several experiments gave curves identical with those in Figure 7. The average amount of factor X_a bound to 1×10^8 platelets was 6×10^{-14} mol. This number was used as the concentration of receptor for the calculation of the bimolecular rate constant of factor X_a–receptor binding. The rate of association was second order within experimental error with respect to factor X_a concentration. The results are given in Table I.

The rate of dissociation of the factor X_a–receptor complex was measured using both the centrifugation assay after addition of a large excess of unlabeled factor X_a and using the filtration assay with and without addition of an excess of unlabeled factor X_a. In experiments where an excess of unlabeled

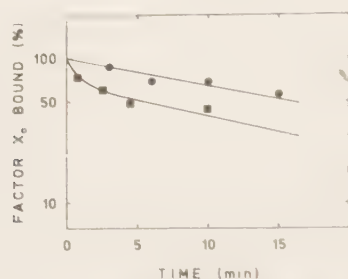


FIGURE 8: Time course of dissociation of specifically bound ^{125}I -labeled factor X_a from platelets (20 ng of labeled factor X_a per 10^8 platelets, equilibrated at 22°C for 25 min). One set of samples was diluted with 40 volumes of incubation buffer at 22°C and at the times indicated the samples were filtered under reduced pressure using $1.2\text{-}\mu\text{m}$ Millipore filters and washed with 10 mL of ice-cold incubation buffer. In another set a 100-fold excess of unlabeled factor X_a was added and the displacement of the labeled factor X_a was studied with the oil centrifugation technique. (■) Oil centrifugation experiment; (●) Millipore filtration experiment.

factor X_a was added, the semilog plot was not linear indicating that the process was not first order. When the process was studied using the filtration assay without added unlabeled factor X_a a linear plot was obtained (Figure 8). The results of the oil centrifugation experiments and those Millipore filtration experiments in which a 100-fold excess of unlabeled factor X_a in the diluent was used gave identical results. The results are presented in Table I.

The apparent dissociation constant K_D from several experiments, calculated from $K_D = K_{-1}/K_{+1}$, was 2.4×10^{-10} to 5.7×10^{-10} M using the K_{-1} from the filtration assay where no excess of unlabeled factor X_a was added. This is in good agreement with the K_D value from the equilibrium experiments.

Interaction of Dip-F-Factor X_a with Platelet Receptor. The rate of binding of Dip-F-factor X_a was equal to or faster than that for factor X_a within experimental error when using both thrombin and calcium ionophore released platelets (Figure 7). This makes the requirement for enzymatic, i.e., proteolytic, modification of a receptor protein before actual binding can occur unlikely.

Both ^{125}I -labeled factor X_a and ^{125}I -labeled-factor X_a were effectively displaced from the receptor by a large molar excess of unlabeled Dip-F-factor X_a . From Figure 7 it is obvious that the rate of dissociation of ^{125}I -labeled-factor X_a from platelets by unlabeled factor X_a and Dip-F-factor X_a was more rapid than the dissociation of ^{125}I -labeled factor X_a . Moreover the dissociation of ^{125}I -labeled Dip-F-factor X_a was more complete than that of ^{125}I -labeled factor X_a . This might have been due to the microscopic aggregation of platelets that was observed late in the incubations with factor X_a ; i.e., the labeled protein might have been mechanically trapped between aggregating platelets to some extent. Such aggregation was not, however, seen in incubations with ^{125}I -labeled Dip-F factor X_a . A 100-fold excess unlabeled $\text{PhCH}_2\text{SO}_2\text{F}$ -factor X_a also displaced ^{125}I -labeled factor X_a from the receptor.

Displacement of factor X_a from platelets by Dip-F factor X_a effectively inhibits thrombin formation in the platelet prothrombin-factor X_a system. In incubations with increasing concentrations of Dip-F factor X_a , the rate of thrombin formation gradually decreased (Figure 9a). If the Dip-F factor X_a was added after the reaction had been started by factor X_a , all further prothrombin activation was effectively inhibited as seen in Figure 9b. This indicates that factor X_a was required not only to initiate the reaction but also for the continued prothrombin activation. From Figure 9a it is obvious that it

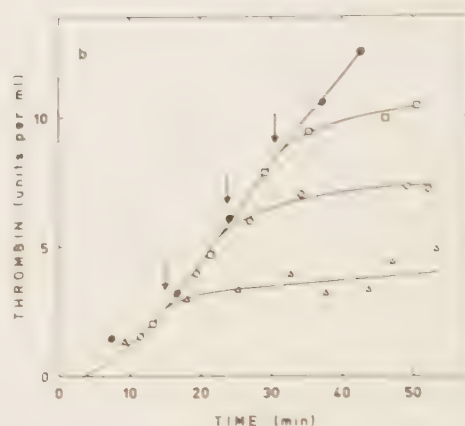
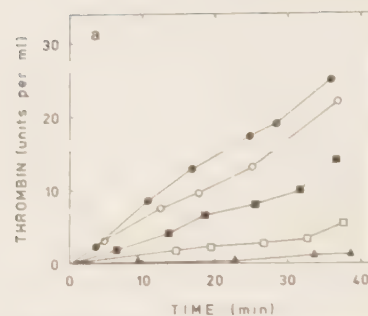


FIGURE 9: Effect of increasing concentrations of Dip-F factor X_a on rates of prothrombin activation. The prothrombin activation was measured in parallel mixtures of platelets (1×10^8 per mL), prothrombin (0.14 mg per mL), and increasing concentrations of Dip-F factor X_a . The reaction was started by the addition of factor X_a (11.8 ng per mL). The reaction mixtures contained the following concentrations of Dip-F-factor X_a (○) 24 ng per mL; (■) 59 ng per mL; (□) 118 ng per mL; (▲) $1.6\text{ }\mu\text{g}$ per mL; (●) control without Dip-F factor X_a . (b) Effect of the addition of an excess of Dip-F-factor X_a on rates of prothrombin activation. The prothrombin activation was measured in parallel mixtures of platelets (1×10^8 per mL), prothrombin (0.14 mg per mL), and factor X_a (11.8 ng per mL). At the times indicated (arrows) a 100-fold excess DIP-factor X_a was added to the different reaction mixtures.

takes about a fivefold molar excess of Dip-F-factor X_a to cause a 50% inhibition of the thrombin formation rate. This is probably due to a somewhat lower association constant for Dip-F factor X_a binding to the receptor than for factor X_a binding. This is also suggested from Figure 7, which shows that both the rates of binding and of dissociation of Dip-F-factor X_a from platelets is not quite identical with the rates of binding and dissociation of factor X_a .

Comparison of Prothrombin Activation Rates in the Presence of Platelets and in the Presence of Phospholipid and Factor V. The rate of prothrombin activation was measured in the presence of platelets and three different concentrations of factor X_a (Figure 10a). A maximum rate of activation was observed after a short lag phase even at the lowest factor X_a concentration. Doubling the platelet concentration led to a shortening of the lag phase but the maximum rate was not increased. The prothrombin concentration was not rate limiting. The activation rates were compared with those obtained using optimum concentrations of phospholipid and factor V (Figure 10b) instead of platelets. In these experiments activation rates increased with increasing factor X_a concentrations. The maximum rate of activation was similar to that found in the platelet system. Increasing the prothrombin concentration did not lead to higher rates of activation. With factor V and phospholipid there was no lag phase which might have been due to the fact that the specific activity of the factor V preparation was not increased by incubation with thrombin. No

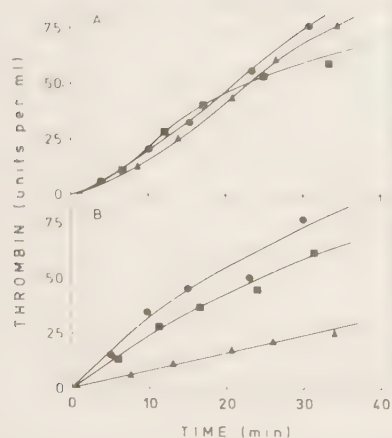


FIGURE 10: Rates of thrombin formation in the presence of platelets and phospholipid and factor V. (A) Prothrombin, 0.14 mg per mL, 2.5 mM CaCl_2 , and platelets 1×10^8 , were incubated with increasing concentration of factor X_a . (B) Same as A except that phospholipid, 0.2 mg per mL, and factor V, 1 unit per mL, were used instead of platelets. The factor X_a concentrations were: (● ●) 34 ng per mL; (■ ■) 17 ng per mL; and (▲ ▲) 3.4 ng per mL.

measurable thrombin activity formed within 40 min if the factor V or the phospholipid was omitted from the incubation mixture. From these experiments it is obvious that the platelet system is more effective in generating thrombin than is phospholipid-factor V at low factor X_a concentrations. This difference is even larger if the factor X_a concentration is further decreased.

Discussion

The factor X preparations were electrophoretically homogenous before and after activation with RVV and had high clotting activity. A small amount of autolysis product that slowly appeared on storage of the active enzyme did not significantly influence the results. The iodinated factor X_a was native as judged by antibody precipitation tests and bound to platelets in a way that was indistinguishable from that of the unlabeled protein. Furthermore the degree of nonsaturable, "nonspecific" binding to platelets was low and fairly constant from one experiment to another and from one factor X_a preparation to another and most importantly binding correlated with the physiological response, i.e., the rate of thrombin formation. With these criteria fulfilled conclusions drawn on the interaction of native factor X_a with platelets from these experiments should be valid.

The interaction between factor X_a and platelets that had undergone the release reaction by incubation with thrombin or calcium ionophore had the properties of a true receptor interaction and had the same general characteristics as the binding of polypeptide hormones to cell surface receptors (Cuatrecasas & Hollenberg, 1976). The binding was saturable and had high affinity, it was reversible, and the binding data correlated well with the biological dose-response curve. This indicates that a biologically significant interaction was studied. The results obtained in this study confirm and extend the results recently reported by Miletich et al. (1977). Thus the number of binding sites per cell was 290–420 which compares well with the value of 380 human factor X_a molecules bound per platelet at saturation reported by these authors. The rate constant for binding of factor X_a to the platelet receptor was comparable to that obtained for binding of polypeptide hormones to cell surface receptors (Cuatrecasas and Hollenberg, 1976; Cuatrecasas, 1971; Frazier et al., 1974). The rate of dissociation of the factor X_a -receptor complex was substan-

tially increased in the presence of unlabeled factor X_a . Such dissociative behavior has also been found for some polypeptide hormone receptor complexes. Plausible explanations for this may be negatively cooperative binding or receptor heterogeneity (Cuatrecasas & Hollenberg, 1976).

In prothrombin the vitamin K dependent calcium binding structures required for phospholipid binding are all in the aminoterminal part of prothrombin fragment 1. The amino acid sequence in this part of the molecule shows an extensive homology to the amino-terminal γ -carboxyglutamic acid containing part of the light chain of factor X. This would indicate that the phospholipid binding of prothrombin and factor X are similar, although some differences have been reported (Nelsestuen & Lim, 1977; Nelsestuen & Broderius, 1977; Lim et al., 1977). It may thus be noted that the association constant for binding of prothrombin fragment 1 to phospholipid in the presence of calcium ions was found to be in the range of 2×10^5 to 10^6 M^{-1} by Dombrose et al. (1978). This should be compared with the association constant for binding of factor X_a to platelets found in our experiments, i.e., approximately $3\text{--}5 \times 10^9 \text{ M}^{-1}$ from both equilibrium measurements and rate measurements. Miletich et al. (1977) could not demonstrate binding of human factor X to platelets and we could not demonstrate binding of bovine factor X to bovine released platelets nor could we demonstrate binding of prothrombin to the platelets (not published). If the two zymogens have association constants for binding to platelets of the same order of magnitude as Dombrose et al. (1978) found for binding of prothrombin fragment 1 to phospholipid, this is not surprising. Furthermore if one assumes that the rate of formation of zymogen-platelet receptor complex is approximately the same as that for factor X_a binding to platelets the half-life of the complex should be less than a second.

The large difference in association constant between prothrombin fragment 1 and phospholipid and factor X_a and platelets would indicate that platelets catalyze prothrombin activation not only by supplying suitable, i.e., negatively charged, phospholipid but is involved in some other way as well. This is in line with the observation of Marcus et al. (1966) that platelet membrane particles are approximately 20 times more effective than a corresponding amount of platelet membrane lipid in the so called thromboplastin generation test. In contrast to this, however, it was not possible in a more recent study (Vecchione & Zucker, 1975) to demonstrate that platelets provide other procoagulant activity in plasma than phospholipid. In this context it is also noteworthy that we found that at very low factor X_a concentrations the platelet system is more effective than a phospholipid-factor V system, whereas at higher factor X_a concentrations the rates of prothrombin activation are comparable.

In addition to factor X_a , calcium ions, phospholipid, and factor V are part of the prothrombin activation complex. Recently factor V coagulant activity was found in platelets by Breederveld et al. (1975) and by Østerud et al. (1977) who also showed that the factor V activity increased when the platelets were lysed by freezing and thawing. The fact that factor X_a binds to factor V and that factor V can be inactivated by thrombin adds to the similarities of factor V and the platelet factor X_a receptor since Miletich et al. (1977) showed that the receptor was inactivated by high concentrations of thrombin. In this context it should be noted that factor V can bind prothrombin and factor X_a only after it has been activated by thrombin (Suttie & Jackson, 1977; Freeman et al., 1977). Binding of factor X_a to platelets, however, does not require thrombin activation since binding is identical to thrombin and calcium ionophore released platelets as also demonstrated by

Miletich et al. (1977). The possibility that the platelet receptor must be proteolytically modified by factor X_a itself before actual binding can occur seems unlikely since Dip-F-inactivated factor X_a bound like the active enzyme. Whether plasma factor V and the platelet factor X_a receptor are chemically related as are for instance plasma factor XIII and platelet factor XIII, or chemically unrelated is, of course, unclear.

Acknowledgment

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The Activation of Prothrombin by Platelet-Bound Factor X_a

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After the platelet release reaction, factor X_a binds to a receptor on the platelet surface with high affinity, K_a 5.2×10^9 M $^{-1}$. Binding of the factor X_a substrate, prothrombin to the platelet surface has not yet been possible to demonstrate. The interaction between prothrombin and platelets was therefore studied by determination of the structural requirements on the prothrombin molecule for activation by platelet-bound factor X_a . A comparison was made between the rates of activation of normal prothrombin, prethrombin 1, prethrombin 2, and acarboxyprothrombin (prothrombin lacking γ -carboxyglutamic acid residues) in the presence of factor X_a and isolated platelets. Both the vitamin-K-dependent fragment 1 of prothrombin and fragment 2 which interacts with factor V were found to be required for rapid prothrombin activation. Acarboxyprothrombin and prethrombin 1 were slowly activated to thrombin by factor X_a on the platelet surface after the platelet release reaction. In the same system prethrombin 2 was activated more slowly to thrombin. In the presence of fragment 2, which binds noncovalently to prethrombin 2, the activation rate was enhanced and equal to that of prethrombin 1 supporting the conclusion that factor V is part of the platelet receptor. The cleavage products of prothrombin by factor X_a in the presence of platelets were found to be the same as those previously described when the activation was catalyzed by the prothrombinase complex. The coefficient for proteolytic efficiency, k_{cat}/K_m , was found to be approximately 50 times higher in the presence of prothrombin than in the presence of acarboxyprothrombin. We conclude that prothrombin interacts with a specific platelet structure consisting of phospholipid and an activated form of factor V exposed on the platelet surface as a result of the release reaction.

The activation of prothrombin by factor X_a is the last zymogen activation in the blood coagulation cascade [1–9]. It involves the cleavage of two peptide bonds (Fig. 1). The rate of activation is greatly enhanced in the presence of the macromolecular prothrombinase complex consisting of factor X_a , phospholipid, factor V and calcium ions. In the prothrombinase complex the following interactions have been demonstrated: factor X_a with Ca^{2+} and phospholipid; factor V with phospholipid; factor X_a with factor V [9, 10]. Prothrombin has an affinity for both the phospholipid and the factor V part of the prothrombinase complex [9]. The binding of prothrombin to the phospholipid requires calcium and is mediated by the γ -carboxyglutamic acid residues in the fragment 1 part of the molecule whereas the fragment 2 part interacts with factor V.

Platelet phospholipid extracts stimulate blood clotting *in vitro*. Platelet lipoprotein extracts are even more effective in this respect [11, 12]. Miletich et al. [13–15] have demonstrated binding of human factor X_a to a receptor on isolated platelets. They found

that platelet-bound factor X_a catalyzed prothrombin activation more readily than factor X_a in a system with optimal concentrations of phospholipid and factor V.

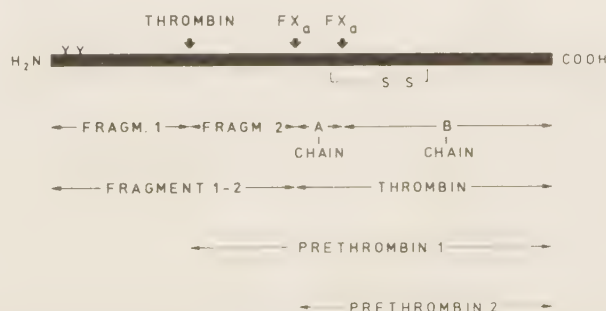


Fig. 1. Schematic diagram of the prothrombin molecule. Two peptide bonds are cleaved by factor X_a forming thrombin and fragment 1–2. Prethrombin 2 and fragment 1–2 are formed if only the first peptide bond is cleaved by factor X_a . Fragment 1–2 is cleaved by thrombin giving rise to fragment 1 and fragment 2. The intact prothrombin molecule can also be cleaved by thrombin forming prethrombin 1 and fragment 1. The symbol (Y) denotes γ -carboxyglutamic acid residues

Factor V is part of the receptor and it becomes accessible only after the release reaction. Recently we characterized the binding of bovine factor X_a to a platelet receptor [16]. Efforts to demonstrate binding of prothrombin have not been successful [16, 17] and little is known about the interaction between prothrombin and platelets.

The purpose of the present study was to investigate the structural requirements on prothrombin for its interaction with platelets. We have therefore compared the rates of activation of prothrombin, prethrombin 1 and prethrombin 2 by factor X_a in the presence of platelets. We also compared the activation rates of prothrombin and acarboxyprothrombin in the same system to elucidate the role of the γ -carboxyglutamic acid residues for the interaction between prothrombin and platelets. Acarboxyprothrombin formed after administration of the vitamin K antagonist dicoumarol does not bind Ca^{2+} ions or phospholipid but interacts with factor V similarly to normal prothrombin [18–24]. Our results indicate that both the vitamin-K-dependent γ -carboxyglutamic acid residues in the fragment 1 part of prothrombin and the factor V binding part, fragment 2, are required for rapid activation of prothrombin on the surface of isolated platelets.

MATERIALS AND METHODS

Materials

Plasma deficient in factors VII and X, Russel's viper venom, the Taipan snake venom (*Oxyuranus scutellatus scutellatus*), Folch fraction III, rabbit brain cephalin and bovine serum albumin (Cohn fraction V) were from Sigma Chemical Co. (St Louis, MO, U.S.A.) and the brain thromboplastin from Ortho Diagnostics (Raritan, NJ, U.S.A.). Hydroxy[^{14}C]tryptamine bioxalate (44 Ci/mol) was from New England Nuclear (Dreieichenhain, F.R.G.). Aprezon oil A was from Shell International Chemical Company Limited (London, England) and di-*n*-butyl-phthalate from the British Drug House (Poole, England). The calcium ionophore A 23187 was kindly supplied by E. Lilly (Stockholm, Sweden). Bovine fibrinogen was from Behringwerke AB (Marburg, F.R.G.). Heparin (5000 IE/ml) was from Vitrum AB (Stockholm, Sweden).

Coagulation Factors

Bovine prothrombin and bovine acarboxyprothrombin (obtained after administration of the vitamin K antagonist dicoumarol to a cow) were purified as described previously [25, 26]. The proteins were dialyzed against 0.050 M Tris-HCl, 0.15 M NaCl, pH 7.5 and stored in aliquots at $-70^\circ C$.

The bovine thrombin used for digestion of prothrombin, to fragment 1 and prethrombin 1, was

prepared by activation of bovine prothrombin with the Taipan snake venom and purified by QAE-Sephadex A-50 chromatography [27]. It had a fibrinogen clotting activity of 620 N.I.H. units/mg protein. High-specific-activity thrombin (2500 N.I.H. units/mg) was prepared by activation of prothrombin with factor X_a in the presence of phospholipid and factor V. It was purified by chromatography on SP-Sephadex as described by Lundblad et al. [28]. This thrombin was used as a reference in sodium dodecylsulphate gel electrophoresis experiments.

Prethrombin 1 and fragment 1 were obtained by thrombin digestion of prothrombin. They were isolated by chromatography on a column (2.5×35 cm) with DEAE-Sephadex A-50 in 0.2 M Tris-HCl, pH 8.0 and eluted with a linear gradient of NaCl (0–0.3 M, 600 ml in each vessel) in the same buffer. Prethrombin 2 and fragment 2 were prepared by activation of prethrombin 1 with factor X_a in 25% trisodium citrate essentially as described by Mann [8]. The activation was terminated by adding bovine antithrombin III (final concentration 0.18 mg/ml) and 50 units heparin/ml. Fragment 2 was isolated by chromatography on a column (1.5×25 cm) with QAE-Sephadex A-50 in 20 mM Tris-HCl, 0.1 M NaCl, pH 7.5 and eluted with a linear gradient of NaCl in the same buffer (0.1–0.6 M, 200 ml in each vessel). The peak containing prethrombin 2 was dialyzed against 0.025 M sodium phosphate, pH 6.5 and chromatographed on a SP-Sephadex G-50 column (1.5×25 cm) in the same buffer and eluted with a linear gradient of sodium phosphate (0.025–0.45 M, 200 ml in each vessel). Fragment 1, fragment 2, prethrombin 1 and prethrombin 2 were homogeneous on sodium dodecylsulphate polyacrylamide electrophoresis. They were dialyzed against 0.025 M Tris-HCl, 0.07 M NaCl, 50% (v/v) glycerol pH 7.5 and stored at $-20^\circ C$.

Bovine factor X was purified as described previously [26]. It was activated with purified factor X activator from Russel's viper venom [29] and purified by DEAE-Sephadex chromatography as outlined by Jesty and Nemerson [30]. The factor X_a was dialyzed against 0.025 M Tris-HCl, 0.07 M NaCl, 50% glycerol pH 7.5 and stored at $-20^\circ C$. Two preparations were made, the first gave a clotting time in the factor X_a assay of 20 s when diluted to a concentration of 11 ng/ml, the second a clotting time of 28 s at a concentration of 17 ng/ml.

Highly purified bovine factor V was a generous gift from Dr Peter Esnouf (Radcliff Infirmary, Oxford, England). The factor V activity of this preparation did not increase further upon incubation with thrombin. Bovine antithrombin III was purified as described by Miller-Andersson et al. [31]. A phospholipid suspension was prepared from Folch fraction III as described previously [16].

The proteins were quantified spectrophotometrically using the following values for $A_{1\text{ cm}}^{1\%}$ at 280 nm: prothrombin and acarboxyprothrombin, 14.6 [32]; prethrombin 1, 19.2; thrombin and prethrombin 2, 21.4; fragment 1, 10.1; fragment 2, 13.8 [4]; factor X, 12.4 [33]; antithrombin III, 6.0 [34].

Platelet Isolation and Determination of the Platelet Release Reaction

Platelets were isolated from bovine blood with a procedure modified from Tollefsen et al. [35]. Within 3–5 min after slaughter nine parts of bovine blood were collected in a plastic bottle containing one part of 2.85% trisodium citrate solution. Platelets isolated from blood collected in this way and from blood drawn by jugular vein puncture gave identical results. Whole blood was centrifuged for 2.5 min at 3200 rev./min (approximately $1000 \times g$) in a Sorvall RC2-B centrifuge at room temperature using the SS-34 rotor to obtain platelet-rich plasma. The platelets were sedimented by centrifugation at the same speed for 10 min instead of at $2250 \times g$ for 15 min as used by Tollefsen et al. This modification, which was used throughout the entire isolation procedure, facilitated resuspension of the platelet pellets. The platelets were labelled with hydroxy [^{14}C]tryptamine binoxalate as described previously [16]. The release reaction was measured with the oil centrifugation technique [13, 36] by determining [^{14}C]serotonin in aliquots of supernatants. 100% release was estimated by counting ^{14}C in an aliquot of the platelet suspension without prior centrifugation. Radioactivity was measured in Packard Instagel in a liquid scintillation counter. In some experiments the release reaction was induced by adding the calcium ionophore A 23 187 to a final concentration of $1\text{--}2\text{ }\mu\text{M}$.

Coagulation Assays

All assays were done in a Fibrometer coagulation timer (BBL). Thrombin activity was determined by the method of Fenton and Fasco [37]. The assay was standardized with a thrombin standard (lot B-3) kindly supplied by Dr D. L. Aronson (Department of Health, Education and Welfare, Bethesda, MD, U.S.A.). Factor X_a was assayed according to Backman et al. [38] using cephalin without Russel's viper venom and factor-VII + X-deficient test plasma. Factor V was assayed by the method of Kappeler [39]. Pooled normal citrated human plasma was defined to have one unit of factor V activity/ml. Factor-V-deficient plasma was prepared by incubating human oxalated plasma at 37°C for approximately 72 h [40].

Prothrombin Activation

All activations, unless noted, were done at room temperature ($22\text{--}24^\circ\text{C}$) in 0.05 M Tris-HCl, 0.15 M

NaCl, pH 7.5 containing 5 mg bovine serum albumin and 1 mg glucose/ml. The final concentration of CaCl_2 in all activations was 2.5 mM. Normal prothrombin, acarboxyprothrombin or prethrombin 1 were incubated with platelets (10^8 ml) or phospholipid ($20\text{ }\mu\text{g}$ ml) and/or factor V (1 unit/ml) and the activations were started by adding factor X_a . Aliquots of $10\text{--}100\text{ }\mu\text{l}$ were removed for the determination of thrombin activity.

Electrophoretic and Immunochemical Methods

In experiments performed to study the conversion products in the presence of platelets no albumin was used in the buffer. Aliquots of the incubation ($200\text{ }\mu\text{l}$) were removed at intervals and centrifuged to remove the platelets. The supernatants were added to $200\text{ }\mu\text{l}$ of 0.05 M Tris-HCl pH 6.8, 2% sodium dodecylsulphate, 0.002 M EDTA, 10% glycerol. No further proteolysis of prothrombin by factor X_a or thrombin could be detected in this buffer. Vertical sodium dodecylsulphate slab gel electrophoresis was performed in 1.5-mm-thick slab gels containing 10% acrylamide and 0.27% *N,N'*-methylenebisacrylamide using the buffer system of Laemmli [41]. Each sample ($50\text{ }\mu\text{l}$) contained $15\text{--}20\text{ }\mu\text{g}$ protein. The gels were stained in 0.05% Coomassie brilliant blue in 40% methanol and 5.3% acetic acid and destained in 20% methanol and 7% acetic acid. The activation products were identified by comparing the electrophoretic mobility of the components with the corresponding isolated proteins.

Crossed immunoelectrophoresis was performed according to Ganrot [42]. Monospecific rabbit antisera against prothrombin fragment 1 and fragment 2 and prethrombin 1 were prepared as described earlier [25].

Characterization of Acarboxyprothrombin, Prethrombin 1 and Prethrombin 2

The acarboxyprothrombin preparation used for comparisons with normal prothrombin should not contain γ -carboxyglutamic acid residues or contaminating normal prothrombin. The γ -carboxyglutamic acid content, determined as described previously [43] was found to be less than 0.2 mol/mol protein. The normal prothrombin preparation contained 11.6 mol γ -carboxyglutamic acid residues/mol protein.

The rates of activation of normal prothrombin and acarboxyprothrombin to thrombin by high concentration of factor X_a were equal within experimental error as shown in Fig. 2. The addition of phospholipid to the incubations increased the normal prothrombin activation rate whereas the activation rate of acarboxyprothrombin was not significantly altered. The factor V binding part, fragment 2, is assumed to be un-

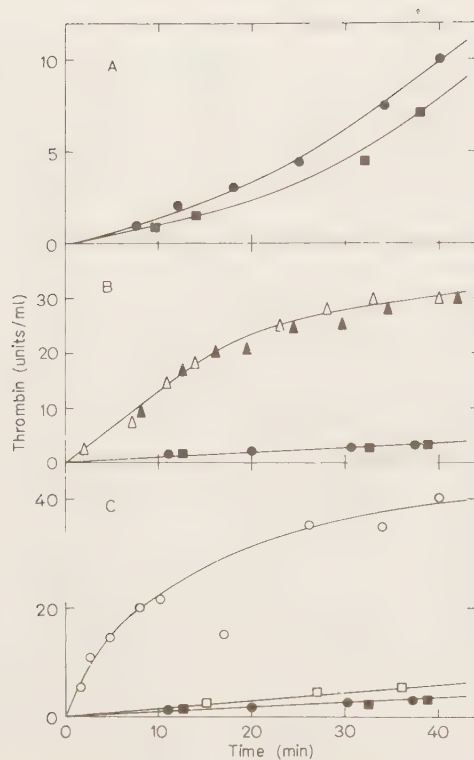


Fig. 2. Activation of normal and acarboxyprothrombin by factor X_a in the absence and presence of factor V or phospholipid. Normal prothrombin (0.15 mg/ml) or acarboxyprothrombin (0.15 mg/ml) and calcium chloride (2.5 mM) were incubated with (A) only factor X_a (10 μ g/ml); (●) normal prothrombin; (■) acarboxyprothrombin. (B) Incubation with factor X_a (4.5 μ g/ml) and factor V (1 unit/ml); (Δ) normal prothrombin; (▲) acarboxyprothrombin; (○, ■) normal and acarboxyprothrombin respectively without the factor V. (C) Incubation with factor X_a (4.5 μ g/ml) and phospholipid (20 μ g/ml); (○) normal prothrombin; (□) acarboxyprothrombin; (●, ■) normal and acarboxyprothrombin respectively without the phospholipid.

altered by vitamin K action during biosynthesis and accordingly the rates of activation of acarboxyprothrombin and prothrombin by factor X_a in the presence of factor V were found to be identical. These results agree with those previously reported [21,22] and indicate that the acarboxyprothrombin was not contaminated with biologically active prothrombin. Activation studies performed in the same system with the preparations of prethrombin 1 and prethrombin 2 also gave results that were very similar to those previously reported [44,45].

RESULTS

Requirement of Both Phospholipid and Factor V Binding Parts of Prothrombin for Rapid Activation in the Presence of Platelets

To determine whether the vitamin-K-dependent structures of prothrombin are required for rapid thrombin generation in the presence of isolated pla-

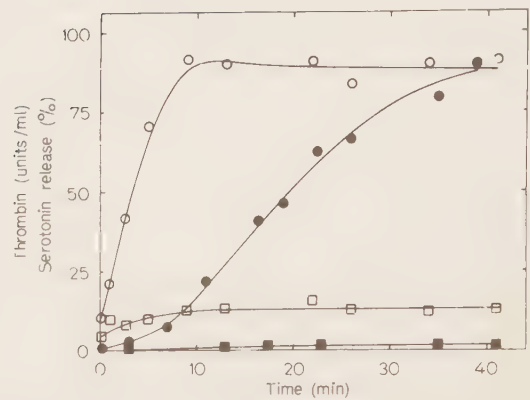


Fig. 3. Platelet-catalyzed prothrombin activation and its relation to serotonin release; differences between normal and acarboxyprothrombin. Serotonin release and thrombin generation were measured (as described in Materials and Methods) in parallel incubations with normal or acarboxyprothrombin (0.15 mg/ml), platelets (10^8 ml), calcium (2.5 mM) and factor X_a (8 ng/ml). The incubations were started with the factor X_a . (○) Serotonin release and (●) thrombin generation during incubation with normal prothrombin. (□) Serotonin release and (■) thrombin generation during incubation with acarboxyprothrombin.

telets, parallel incubations were performed with normal prothrombin and acarboxyprothrombin. Serotonin release and thrombin generation were measured. Following the addition of factor X_a , serotonin was rapidly released in the incubation containing normal prothrombin and after a short lag phase the thrombin generation rate was maximal (Fig. 3). During the lag phase factor X_a was bound to the platelet receptors (not shown in the figure). In incubations with acarboxyprothrombin no thrombin was generated during the first 30–50 min and the serotonin release was small. Crossed immunoelectrophoresis also demonstrated that the acarboxyprothrombin remained intact at this stage of the incubation. If the incubation was further extended a slow thrombin generation was found, preceded by a slow serotonin release. Addition of the releasing agent A 23187 (1 μ M) to the incubation with acarboxyprothrombin shortened the lag phase before thrombin generation started, presumably due to exposure of the factor X_a receptor with which the fragment 2 part of acarboxyprothrombin could interact.

To elucidate the importance of the fragment 1 and fragment 2 parts, the rates of activation of prothrombin, prethrombin 1 and prethrombin 2 by factor X_a in the presence of released platelets were compared. It can be seen from the activation curves (Fig. 4) that prethrombin 1 was converted to thrombin approximately three times faster and prothrombin ten times faster than prethrombin 2. The activation rate of prethrombin 2 was enhanced by the addition of fragment 2 and at equimolar concentrations the activation rate was identical with that of prethrombin 1. It can

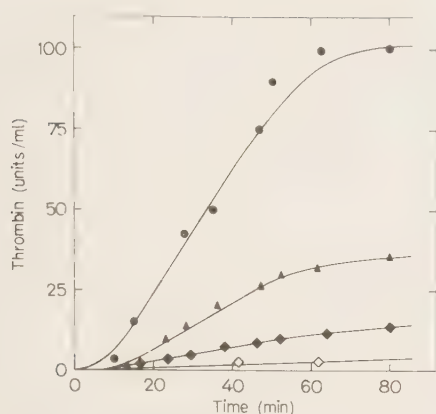


Fig. 4. Activation of normal prothrombin, prethrombin 1 and prethrombin 2 by factor X_a in the presence of platelets after induction of the release reaction. In parallel incubations with platelets (10^8 /ml), calcium chloride (2.5 mM) and equimolar concentrations of prothrombin (0.22 mg/ml, $3 \mu\text{M}$), prethrombin 1 (0.15 mg/ml, $3 \mu\text{M}$) and prethrombin 2 (0.11 mg/ml, $3 \mu\text{M}$) release reaction were induced by adding the calcium ionophore A 23187 to a final concentration of $1 \mu\text{M}$. After 5 min the activations were started by adding factor X_a (200 ng/ml). (●) Normal prothrombin; (▲) prethrombin 1; (◆) prethrombin 2; (◇) a parallel incubation with prethrombin 2 without platelets

therefore be concluded that both the fragment 1 and the fragment 2 part of prothrombin are important for rapid prothrombin activation in the presence of platelets.

Prothrombin, Prethrombin 1 and Acarboxyprothrombin are Activated on the Platelet Surface

Thrombin-formation rates in parallel platelet incubations with equimolar concentrations of prothrombin, acarboxyprothrombin or prethrombin 1 and different factor X_a concentrations are shown in Fig. 5. The rates of thrombin formation were estimated from the part of the thrombin-formation curve having maximal slope. The activation rates were a function of the amount of factor X_a bound to the platelet receptors. As shown in Fig. 5 the thrombin-formation rates were maximal in incubations with a factor X_a concentration of 5–10 ng/ml which saturates the platelet factor X_a binding sites [16]. Higher factor X_a concentrations did not further increase the thrombin formation rates indicating that not only normal prothrombin but also acarboxyprothrombin and prethrombin 1 were activated on the platelet surface. The maximal thrombin formation rate with normal prothrombin was about four times greater than with prethrombin 1 and ten times the rate obtained with acarboxyprothrombin. The thrombin-formation rates in incubations with prethrombin 1 were consistently found to be higher than those obtained in parallel incubations with acarboxyprothrombin. The reason for this is unknown.

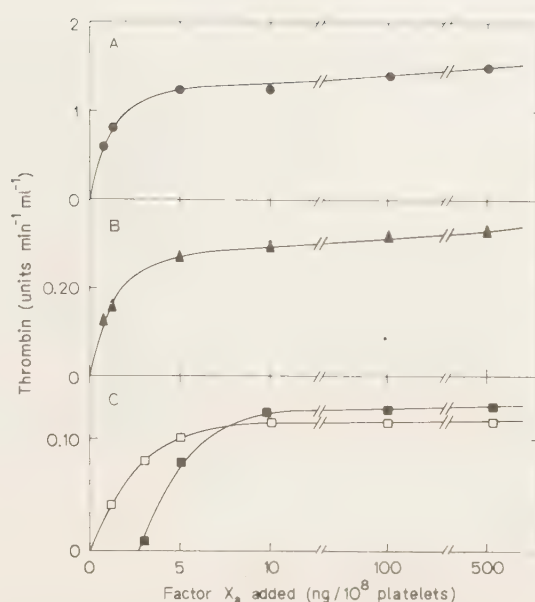


Fig. 5. Thrombin-formation rates in platelet incubations with increasing concentrations of factor X_a . Thrombin generation was measured in parallel incubations with platelets (10^8 ml), calcium chloride (2.5 mM) and (A) normal prothrombin (0.15 mg/ml, $2 \mu\text{M}$), (B) prethrombin 1 (0.10 mg/ml, $2 \mu\text{M}$), (C) acarboxyprothrombin (0.15 mg/ml, $2 \mu\text{M}$) and different factor X_a concentrations. The thrombin formation rates were calculated from the maximal slope of the thrombin formation curves. (●) Normal prothrombin; (▲) prethrombin 1; (□) acarboxyprothrombin; (■) acarboxyprothrombin with the release reaction induced by the calcium ionophore A 23187

Thrombin Yield and Conversion Products in the Presence of Platelets

The thrombin yield in different incubations with factor X_a (20 ng/ml), prothrombin (0.15 mg/ml), and platelets (10^8 ml) was only between 20% and 50% of the theoretical, assuming a specific activity of 2500 U/mg pure bovine thrombin [46]. Both the final yield and the initial activation rate increased with increasing platelet concentrations due to a larger fraction of factor X_a bound. Increasing the factor X_a concentration above the saturating level (5–10 ng/ 10^8 platelets) did not increase the final yield.

The conversion products of prothrombin, prethrombin 1 or acarboxyprothrombin were identified with polyacrylamide gel electrophoresis (Fig. 6). The prothrombin conversion products were identical with those previously described in experiments with phospholipid and factor V (see for instance [4]). Fragment 2 migrated with the tracking dye in the 10% polyacrylamide gels used, but was easily identified by crossed immunoelectrophoresis using a monospecific antiserum against fragment 2. There was a rapid appearance of prethrombin 1 and fragment 1 formed by the action of thrombin on prothrombin. The prothrombin concentration, however, was four times higher in this experiment than in standard incubations to ensure visi-

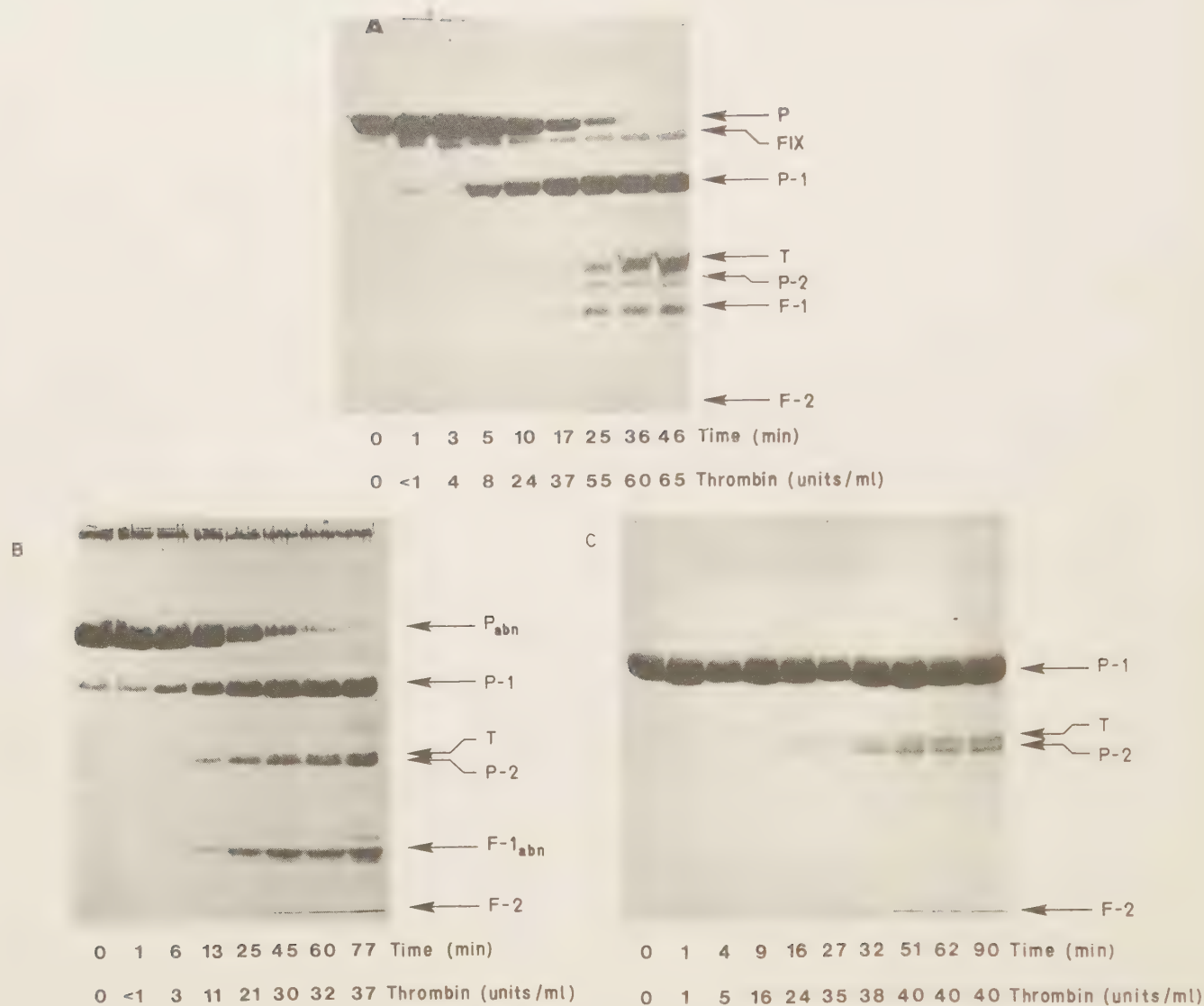


Fig. 6. Conversion products of normal prothrombin, acarboxyprothrombin and prethrombin 1. Platelets (10^8 ml), factor X_a (200 ng/ml), calcium chloride (2.5 mM) and (A) prothrombin (0.69 mg/ml, 9.5 μ M), (B) acarboxyprothrombin (0.69 mg/ml, 9.5 μ M), (C) prethrombin 1 (0.69 mg/ml, 14 μ M) were incubated. The release reaction was induced with the calcium ionophore A 23187 and thrombin was measured. At times indicated aliquots were removed and subjected to slab gel electrophoresis as described in Materials and Methods. In the gels presented the samples were unreduced. P, prothrombin; P_{abn}, acarboxyprothrombin; P-1, prethrombin 1; T, thrombin; P-2, prethrombin 2; F-1, fragment 1; F-1_{abn}, fragment 1 from acarboxyprothrombin; F-2, fragment 2; F-2_{abn}, fragment 2 from acarboxyprothrombin. The high-molecular-weight contaminant in the acarboxyprothrombin samples came from the platelets.

bility of the bands. This substrate concentration, which was approximately six times the concentration required for maximal thrombin-formation rate, favoured thrombin-mediated proteolysis and the amounts of prethrombin 1 and fragment 1 formed were considerably higher than in standard incubations. Prethrombin 2 seemed to be formed to the same extent as thrombin. In incubations with acarboxyprothrombin the same conversion products were found as in normal prothrombin incubations. In the incubation with prethrombin 1 only a relatively small amount (less than 10%) of the substrate was cleaved even after prolonged

incubation. Thrombin generation ceased, even though the substrate concentration was still high, indicating that the factor X_a platelet receptor complex was destroyed presumably by thrombin.

Determination of Kinetic Parameters

To compare the factor X_a substrates normal prothrombin, acarboxyprothrombin and prethrombin 1, the kinetic parameters were measured (Table 1). Thrombin generation rates were measured in parallel incubations of the substrates. Relatively high concen-

Table 1. Kinetic constants for prothrombin activation in the presence of platelets
The average value is given with the range in parenthesis from five determinations using prothrombin, three using prethrombin 1 and two using acarboxyprothrombin. Thrombin units were converted to picomoles by assuming a specific activity of 2500 units mg pure thrombin [46]
 k_{cat} and k_{cat}/K_m were calculated from the mean values of K_m and V

Substrate	K_m	V	k_{cat}	k_{cat}/K_m
	μM	$pM\ s^{-1}$	s^{-1}	$s^{-1}\ \mu M^{-1}$
Prothrombin	0.6 (0.4–0.8)	600 (220–900)	10	16.7
Prethrombin 1	3.6 (2.9–4.4)	180 (80–340)	3.0	0.83
Acarboxyprothrombin	3.6 (3.6–3.6)	80 (30–130)	1.3	0.36

trations of factor X_a (about $100\ ng/10^8$ platelets) were used to ensure rapid platelet release and saturation of the factor X_a receptors. Identical results were found if the platelet-release was induced with the calcium ionophore A 23187. The rates of prothrombin activation were estimated from the maximal slope of the thrombin formation curves when the amount of prethrombin 1 formed was still insignificant. The data plotted according to Lineweaver-Burk always gave linear plots (Fig. 7). We therefore found it justifiable to calculate the apparent K_m and V . The saturating amount of factor X_a has previously been determined to $0.06\ pmol/10^8$ platelets [16]; this was used as total enzyme concentration, $[E]_t$, in the calculations of k_{cat} . Determination of factor X_a binding with ^{125}I -labelled factor X_a and thrombin formation in the same incubation was not done because a decrease in enzymatic activity after labelling of factor X_a had been noticed previously [16]. Active-site titration of factor X_a was not performed and the k_{cat} calculated are minimum values assuming that 100% of bound factor X_a was active. The apparent K_m values for prethrombin 1 or acarboxyprothrombin were equal and about six times higher than that for prothrombin. k_{cat} calculated for prothrombin was higher than k_{cat} for prethrombin 1 and acarboxyprothrombin. The coefficient for proteolytic efficiency, k_{cat}/K_m , was approximately 50 times higher for normal prothrombin than for acarboxyprothrombin.

DISCUSSION

Our present knowledge of the molecular mechanisms involved in activation of prothrombin by factor X_a is the result of detailed studies using the purified accessory components phospholipid, calcium ions, and factor V (for review see [9]). However, relatively little has been known about the physiologically important interaction between platelets and the proteins participating in prothrombin activation. It was therefore an important step forward when Miletich et al. [13] reported that the platelet surface after the release reaction has a specific receptor for human factor X_a .

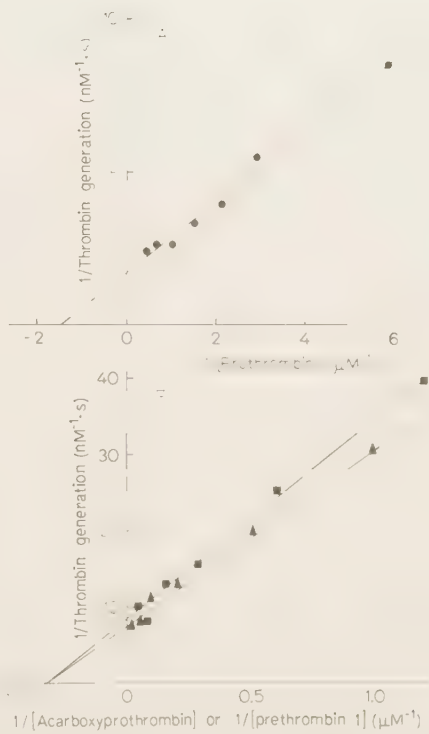


Fig. 7. Lineweaver-Burk plots for the activation of prothrombin, acarboxyprothrombin and prethrombin 1 by factor X_a in the presence of platelets. Thrombin-generation rates were measured in parallel incubations with platelets (10^8 ml), factor X_a ($100\ ng$ ml), calcium chloride ($2.5\ mM$) and different concentrations of (A) (●) prothrombin, (B) (■) acarboxyprothrombin and (▲) prethrombin 1

We have characterized the receptor for platelet factor X_a using bovine materials [16]. Factor X_a bound to the platelet receptor catalyzes the activation of prothrombin very effectively. At low factor X_a concentrations platelets are even more effective than optimal concentrations of phospholipid and factor V and at least 1000-fold more effective than factor X_a and phospholipid alone in prothrombin activation [13,14,16]. We have now found that platelet-bound factor X_a catalyzes the activation of prothrombin in the same way as the traditional prothrombinase complex and the prothrombin conversion

products are identical to those previously identified in the presence of the prothrombinase complex [9]. Furthermore the structural requirements on prothrombin for its activation by platelet-bound factor X_a or by the prothrombinase complex are the same. Thus both fragment 1 (the phospholipid binding part of prothrombin) and fragment 2 (the part that interacts with factor V) are required for rapid activation in both systems. The γ -carboxyglutamic acid residues in prothrombin are also essential for rapid activation, indicating that the interaction between prothrombin and platelet phospholipid is important. From these data we conclude that prothrombin interacts with a specific platelet structure consisting of both suitable phospholipid and of factor V. However, efforts to demonstrate direct binding of prothrombin to platelets with the technique used to measure factor X_a binding have been unsuccessful, probably due to a comparatively low association constant [16, 17].

As early as 1948, Ware et al. [47] reported that platelets possessed factor V activity. Hjort et al. [48] reported that the platelet factor V activity was plasma factor V tightly bound to the platelet surface. More recently, Breederveld et al. [49] and Østerud et al. [50] showed that factor V was located within the platelets and appeared after freezing and thawing or stimulation with collagen. Several pieces of evidence support the conclusion that factor V is part of the factor X_a receptor [13–15]. Both our previous data [16] and those of Miletich et al. [13–15] are consistent with the concept that factor V is located within platelets and appears in an activated form as a result of the release reaction. Thus, an acquired antibody against human factor V blocked the factor X_a binding only when incubated with thrombin-treated platelets but had no inhibitory effect on the subsequently measured factor X_a binding when incubated with intact platelets [14]. Furthermore, the factor V activator from Russell's viper venom cannot induce factor X_a binding sites on the surface of intact platelets (unpublished work). Whether platelet factor V is present in an activated form or an unactivated form that is activated during the platelet release reaction by a platelet factor V activator [50] is unclear.

The K_m of 0.6 μ M prothrombin determined in the presence of platelets was approximately one sixth of that for prothrombin 1 and acarboxyprothrombin. The coefficient for proteolytic efficiency k_{cat}/K_m was approximately 50 times higher in the presence of prothrombin than in the presence of acarboxyprothrombin, illustrating the importance of the γ -carboxyglutamic acid residues. The apparent K_m of 0.6 μ M prothrombin is below the plasma prothrombin concentration (approximately 1.5–3 μ M). It thus appears that the rate of prothrombin activation *in vivo* is relatively uninfluenced by the prothrombin plasma concentration. Silverberg et al. [51] recently reported a K_m

of 0.34 μ M for factor X activation by factor VII_a and tissue factor. The plasma factor X concentration is approximately 0.16 μ M which suggests that the factor X concentration is more important for the regulation of the overall rate of blood clotting. However, very little is yet known about the regulation of blood coagulation *in vivo*.

From the available data we conclude that platelets are important for a localized activation of prothrombin *in vivo*. After adhesion to subendothelial collagen, platelets expose a receptor consisting of an activated form of factor V and suitable phospholipid. Factor X_a binds with high affinity whereas prothrombin binds with lower affinity effectively exposing susceptible bonds for factor X_a . This results in a rapid local thrombin formation.

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Inhibitory Effect of Activated Protein C on Activation of Prothrombin by Platelet-Bound Factor X_a

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Factor X_a binds to a receptor available on the platelet surface after the release reaction. The receptor consists of phospholipid and factor V. Factor X_a bound to the receptor catalyses the activation of prothrombin effectively. The effects of bovine protein C, a vitamin-K-dependent zymogen of a serine protease, on prothrombin activation by platelet-bound bovine factor X_a has been studied. Protein C was found to be activated (protein C_a) by thrombin formed in the prothrombin-platelet-factor X_a incubation. Protein C_a in contrast to the zymogen, protein C, or protein C_a inactivated with diisopropylphosphorofluoridate inhibited prothrombin activation by factor X_a in the presence of platelets. Protein C_a was found to destroy the receptor by proteolysis whereas direct binding of protein C_a to the receptor could not be demonstrated. The inhibition by protein C_a could be monitored as a parallel decrease in factor X_a binding and prothrombin activation. The receptor was protected by factor X_a from proteolysis by protein C_a. Protein C_a was also found to inhibit the interaction between prothrombin and the factor X_a platelet receptor. These results indicate that protein C after activation may have a role as a regulator of prothrombin activation *in vivo*.

Protein C is a vitamin-K-dependent zymogen of a serine amidase isolated from both bovine and human plasma [1, 2]. It has two polypeptide chains with molecular weights of approximately 41 000 and 21 000 [2, 3]. There is a strong homology between the amino acid sequence of bovine protein C and those of the other vitamin-K-dependent proteins, particularly with factor X [4, 5]. The concentration of protein C in bovine plasma is approximately 5 mg/l [1]. Protein C can be activated by the factor-X activator from Russell's viper venom or by thrombin [3, 6]. Protein C_a (activated protein C) is identical with autoprothrombin II-a described by Seegers et al. [7]. Once activated protein C has anticoagulant properties due to inactivation of plasma factor V [6]. Canfield et al. [8] recently reported that factor V_a (factor V treated with a small amount of thrombin) is more susceptible than factor V to proteolysis by protein C_a. Factor V_a is part of the macromolecular prothrombinase complex which likewise consists of phospholipid, calcium ions, and factor X_a. When factor X_a is part of this complex

it effectively catalyses the conversion of prothrombin to thrombin [9]. Isolated platelets can replace factor V_a and phospholipid in the prothrombinase complex [10]. It has recently been shown that induction of the platelet release reaction in isolated platelets leads to the appearance of a receptor for Factor X_a on the platelet surface [11–13]. The receptor consists of factor V_a and phospholipid.

This paper reports that bovine protein C is activated during prothrombin activation by factor X_a bound to platelets. Furthermore protein C_a destroys the platelet receptors for factor X_a and inhibits the interaction between platelets and prothrombin thereby acting as an inhibitor of prothrombin activation. This sequence of events may be a physiologically important regulatory mechanism of blood coagulation *in vivo*.

MATERIALS AND METHODS

Materials

Plasma deficient in factors VII and X, Russell's viper venom, diisopropylphosphorofluoridate (iPr₂P-F), rabbit brain cephalin and bovine serum albumin (Cohn fraction V) were from Sigma Chemical Co. (St Louis, Missouri); di-*n*-butylphthalate was

A preliminary report of parts of this work was presented at the eighth Steenbock symposium, June 1979, in Madison, Wisconsin.

Abbreviations. iPr₂P-F, diisopropylphosphorofluoridate; protein C_a, activated protein C; factor V_a, factor V treated with a small amount of thrombin; iPr₂P-protein C_a, protein C_a inactivated with iPr₂P-F.

from the British Drug Houses (Poole, Great Britain) and Apiezon A oil from Shell International Chemical Co. Ltd (London, Great Britain). 5-Hydroxy[2- ^{14}C]-tryptamine binoxalate (44 Ci/mol) was from New England Nuclear (Dreieichenhain, Federal Republic of Germany) and Na^{125}I from the Radiochemical Center (Amersham, Great Britain). Bovine fibrinogen was from Behringwerke AG (Marburg, Federal Republic of Germany). The chromogenic substrate S-2160 was obtained from Kabi Diagnostica (Stockholm, Sweden).

Bovine prothrombin, factor X, and protein C were purified as described previously [1]. Factor X was activated with the factor-X activator from Russel's viper venom [14] and then isolated by DEAE-Sephadex chromatography [15]. Bovine thrombin was isolated essentially according to Lundblad et al. [16]. It had a fibrinogen clotting activity of 2500 units/mg. Protein C was activated with the factor-X activator from Russel's viper venom or with thrombin [3,6]. It was then isolated by chromatography on a column (1.6×25 cm) with DEAE-Sephadex A-50 in 50 mM Tris-HCl, 0.15 M NaCl pH 7.5 and eluted with a linear gradient of NaCl (0.15–0.55 M, 200 ml in each vessel). All purified proteins were homogenous as judged by 10% polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate using the buffer system of Laemmli [17]. Protein C_a had a specific amidase activity of 4–5 μmol hydrolyzed $\text{min}^{-1} \text{mg}^{-1}$ when tested with the substrate S-2160, which was in good agreement with results previously reported [6]. Protein C_a was stable for more than a year at -70°C in 50 mM Tris-HCl, 0.15 M NaCl, pH 7.5. Protein C_a (0.3 mg/ml) was inactivated with $\text{iPr}_2\text{P-F}$ at a final concentration of 10 mM in 50 mM Tris-HCl, 0.15 M NaCl pH 7.5 at 22°C for 2 h and then dialyzed against the same buffer without $\text{iPr}_2\text{P-F}$. The inactivated protein ($\text{iPr}_2\text{P-protein } C_a$) had no amidase activity.

Methods

All clotting assays were done in a Fibrometer coagulation timer (BBL). Factor X_a activity was measured with plasma deficient in factor VII and X [18] and cephalin without Russel's viper venom. Thrombin was assayed with the method of Fenton and Fasco [19] standardized against a thrombin standard (lot B-3 kindly supplied by Dr D. L. Aronson at the Department of Health, Education and Welfare, Bethesda, Maryland).

Factor X_a , protein C and protein C_a were labelled with ^{125}I by the lactoperoxidase method [20]. Platelets were isolated from bovine blood with a modification [21] of the method of Tollefsen et al. [22]. The isolated platelets were incubated with [^{14}C]serotonin and the release reaction and binding of proteins to

platelets were measured with an oil centrifugation technique as described previously [13].

In experiments performed to ascertain whether labelled protein C was activated during prothrombin activation aliquots (100 μl) were drawn from the platelet reaction mixture at regular intervals and added to 100 μl 50 mM Tris-HCl, 2% sodium dodecylsulphate, 2 mM EDTA, 10% glycerol pH 6.8. After reduction of the samples with 5% 2-mercaptoethanol, vertical sodium dodecylsulphate slab gel electrophoresis was performed in 1.5-mm-thick slab gels containing 10% acrylamide and 0.27% N,N' -methylenebisacrylamide [17].

RESULTS

Protein C_a Inhibits the Activation of Prothrombin by Factor X_a in the Presence of Platelets

Addition of factor X_a to a reaction mixture containing isolated platelets, prothrombin, and calcium ions leads to the formation of only a very small amount of thrombin (presumably on the surface of damaged platelets) which is necessary to induce the platelet release reaction. After the release reaction, factor X_a binds to the platelet surface and prothrombin is rapidly activated to thrombin [11]. When protein C_a was added to the reaction mixture at the same time as factor X_a , the rate of activation of prothrombin was lower although this effect required a rather high concentration of protein C_a (more than 1 $\mu\text{g/ml}$) (Fig. 1). Protein C_a did not appear to influence the platelet release reaction or the length of the lag phase preceding the phase of rapid thrombin formation. The zymogen, protein C, had no effect on the prothrombin activation rate.

Proteolytic Destruction of Factor X_a Receptor Sites by Protein C_a

To find out whether protein C_a acts as a competitive inhibitor of factor X_a binding to the platelet surface, or destroys the factor X_a receptors by proteolysis, platelets (pretreated with 1 unit thrombin/ml to induce the release reaction and thereby to expose factor X_a receptor sites) were incubated with protein C_a before the addition of prothrombin and labelled factor X_a (Fig. 2). The preincubation with protein C_a led to an approximately parallel decrease in factor X_a binding and in initial prothrombin activation rate. Both decreased with increasing duration of preincubation. In the reaction mixture without protein C_a this time-dependent inhibitory effect on factor X_a binding and initial prothrombin activation rate was not observed. When a lower protein C_a concentration was used (0.1 $\mu\text{g/ml}$) somewhat longer

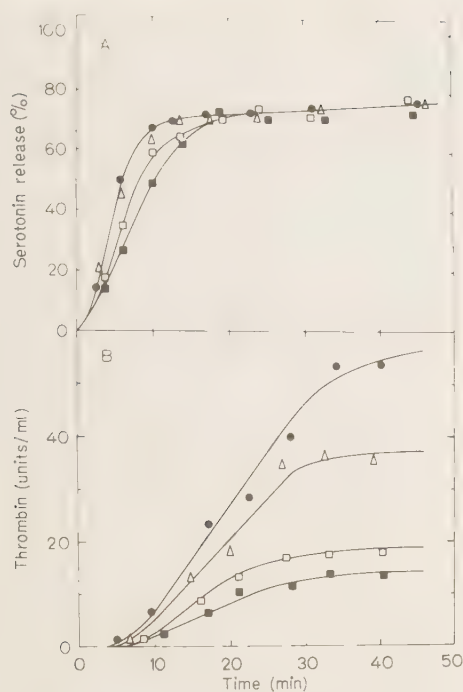


Fig. 1. Effect of protein C_a on prothrombin activation by factor X_a in the presence of platelets and on the platelet release reaction. Platelets (10⁸ ml) labelled with [¹⁴C]serotonin were incubated at 37 °C with prothrombin (0.15 mg/ml) in 50 mM Tris-HCl, 0.15 M NaCl, 5 mM CaCl₂ pH 7.5 containing bovine serum albumin (5 mg/ml) and glucose (1 mg/ml). At zero time the reactions were started by addition of factor X_a (20 ng/ml) and a varying amount of protein C_a. Platelet serotonin release (A) and thrombin formation (B) were followed. Protein C_a concentration in incubation: (●) 0, (Δ) 1 μg/ml, (□) 5 μg/ml, (■) 10 μg/ml

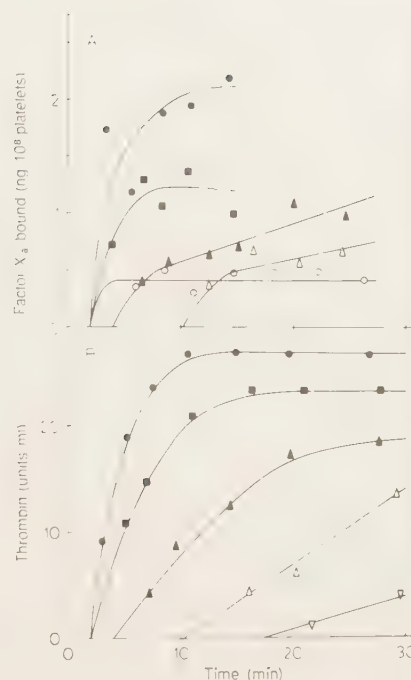


Fig. 2. Effect of protein C_a on the platelet factor X_a receptors and the activation of prothrombin. A platelet suspension (10⁸ ml) was treated with 1 unit thrombin ml and then incubated with 1 μg protein C_a ml at 37 °C. At intervals prothrombin (0.1 mg/ml) and labelled factor X_a (20 ng/ml) were added to aliquots of the incubation and factor X_a binding (A) and thrombin formation (B) were measured. (●) Incubation without protein C_a; (■) protein C_a. ¹²⁵I-labelled factor X_a and prothrombin added simultaneously; (▲) 2-min preincubation; (Δ) 8-min preincubation; (▽) 15-min preincubation; (○) nonspecific binding of factor X_a

preincubation was necessary to obtain the same inhibitory effect. This effect on the factor X_a binding was not noticed when the release reaction had not been induced before the incubation with protein C_a. Furthermore no effect on the factor X_a binding or on the prothrombin activation was noticed when platelets, before or after the release reaction, were incubated with high concentrations of the zymogen, protein C, or iPr₂-P-protein C_a. These results indicate that the receptors for factor X_a were destroyed by proteolysis, i.e. the active site on protein C_a was a prerequisite.

Prothrombin was not included in the experiment illustrated in Fig. 3, to avoid effects that thrombin and prothrombin activation fragments may exert on factor X_a binding. In the reaction mixture containing protein C_a the initial amount of factor X_a bound was found to decline very slowly indicating that, when occupied by factor X_a, the receptors were protected from further proteolysis by protein C_a. When protein C_a was added after saturation of the factor X_a receptor sites (not shown) factor X_a binding decreased relatively slowly, supporting the conclusion that the factor X_a receptors were protected when occupied by factor X_a.

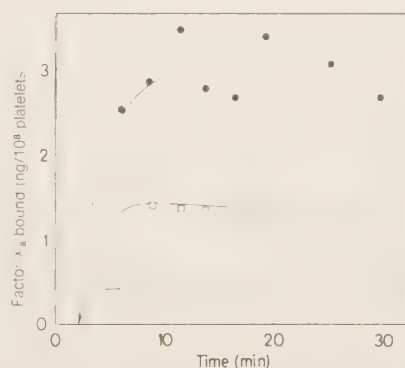


Fig. 3. Protection of the receptors by factor X_a from further proteolysis by protein C_a. Thrombin (1 unit/ml) was added to a platelet suspension (10⁸ ml) at 37 °C. After 2 min, protein C_a (1 μg/ml) and labelled factor X_a (20 ng/ml) were added and factor X_a binding was followed. Note that no prothrombin was added. (●) Incubation without protein C_a; (□) incubation with both ¹²⁵I-labelled factor X_a and protein C_a; (○) nonspecific binding of factor X_a in the absence of protein C_a

Inhibitory Effect of Protein C_a on the Interaction between Prothrombin and Platelets

As already mentioned, the amount of factor X_a bound to platelet receptor was almost constant

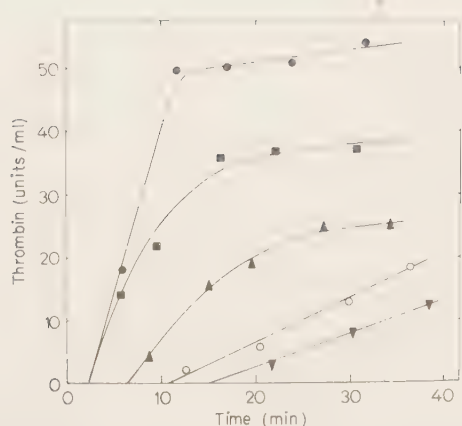


Fig. 4. Destruction by protein C_a of the interaction between prothrombin and platelets. Platelets were treated with thrombin (1 unit/ml) to induce the release reaction and then incubated with 125 I-labelled factor X_a (20 ng/ml) and protein C_a (1 μ g/ml) at 37 °C. At intervals prothrombin (0.1 mg/ml) was added to aliquots of the suspension, and thrombin formation was measured. (●) Incubation without protein C_a ; (■) 125 I-labelled factor X_a , protein C_a and prothrombin added simultaneously; (▲) prothrombin added after 3 min, (○) after 7 min, (▼) after 12 min.

during the incubation when prothrombin was omitted and protein C_a and labelled factor X_a were added simultaneously. This made it possible to find out whether protein C_a inhibited the interaction between platelets and prothrombin. Protein C_a and labelled factor X_a were therefore added to a suspension of platelets pretreated with thrombin, and the factor X_a binding was measured. At intervals prothrombin was added to aliquots drawn from the reaction mixture and thrombin formation was followed. No difference in the amount of factor X_a bound was found in aliquots drawn at different times. Yet the rate of prothrombin activation gradually decreased (Fig. 4) indicating that the inhibitory effect of protein C_a was not merely mediated by decreasing the factor X_a binding, but also by inhibiting the interaction between prothrombin and platelets. When, at regular intervals, prothrombin was added to a control mixture without protein C_a , no difference in initial prothrombin activation rate was observed.

Specific binding of labelled protein C or C_a to platelets could not be demonstrated, whether the release reaction had been induced or not. A wide range of protein C_a concentrations (0.01–10 μ g/ml) was used in these experiments.

Activation of Protein C during Prothrombin Activation in the Presence of Platelets

During the activation of protein C an amino-terminal activation peptide (14 amino acid residues long) is released from the heavy chain [3]. This can be monitored on polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate (Fig. 5). This technique was used to determine whether 125 I-labelled

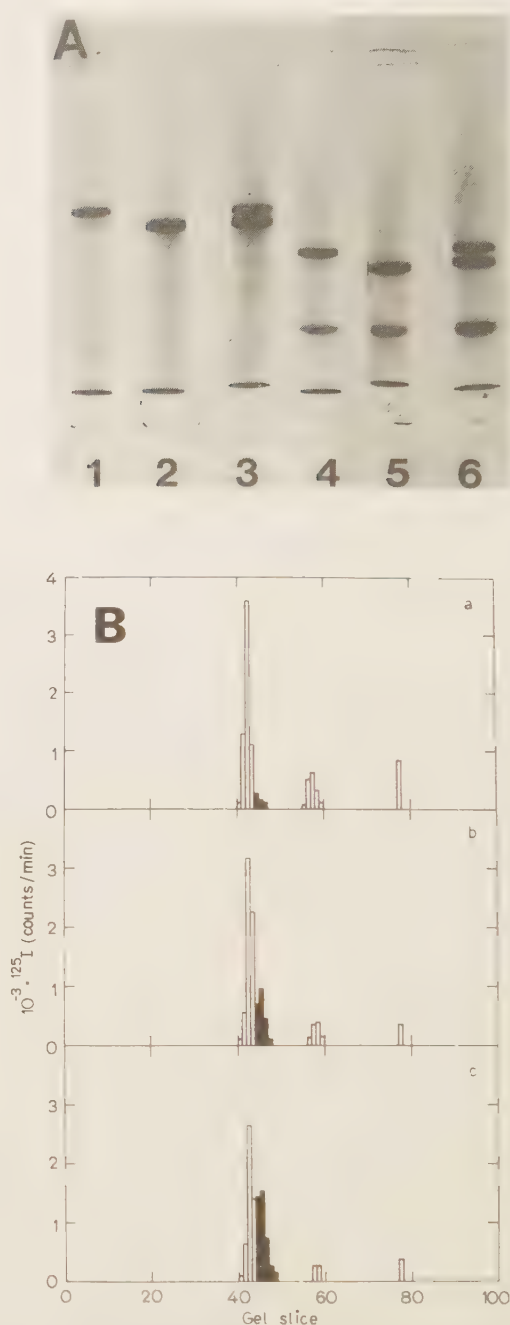


Fig. 5. (A) Sodium dodecylsulphate polyacrylamide (10%) disc gel electrophoresis of bovine protein C and protein C_a . (B) Activation of protein C during thrombin formation in the presence of platelets. (A) (1–3) Unreduced samples; (4–6) samples reduced with 5% 2-mercaptoethanol; (1, 4) protein C; (2, 5) protein C_a ; (3, 6) mixture of protein C and protein C_a , 10 μ g (1, 2, 4, 5) or 20 μ g (3, 6) was loaded on each gel. Tracking dye is seen at the bottom of each gel. (B) 125 I-labelled protein C (5 μ g/ml) was incubated at 37 °C with platelets (10^8 ml) and prothrombin (0.15 mg/ml). The reaction was started by the addition of factor X_a (100 ng/ml) and thrombin generation was followed. At intervals, aliquots were drawn, reduced, and subjected to slab-gel electrophoresis. The radioactivity was measured in 1-mm slices of the gel. From left to right, slices correspond to the heavy chain (filled bars indicate the activated form), the light chain, and free iodine. (a) Before, (b) after 20 min and (c) after 60 min of incubation.

protein C was activated during prothrombin activation in the presence of platelets. As shown in Fig. 5 approximately 20% of the added protein C, corresponding to 1 µg/ml, was found to be activated after a 20-min incubation. By this time approximately 30–35 units thrombin had been formed. After 60 min 40–45 units thrombin were generated and the activated form of protein C accounted for approximately 40%.

DISCUSSION

Platelets contain an activated factor V [23,24], which is part of the recently characterised receptor for factor X_a [11–13,25]. Factor X_a bound to the receptors catalyses the activation of prothrombin effectively. During prothrombin activation, prothrombin interacts with both the factor V_a part and the phospholipid part of the factor X_a receptor and thereby exposes susceptible bonds in prothrombin to factor X_a [21]. We have now shown that protein C_a inhibits the activation of prothrombin by factor X_a in the presence of platelets. This inhibition is probably due to limited proteolysis of platelet factor V_a resulting in a decreased binding of factor X_a and an impaired interaction with prothrombin. The factor X_a receptors are presumably important for local activation of prothrombin in primary hemostasis. The destruction of these receptors by protein C_a may therefore be an important mechanism in the regulation of blood coagulation *in vivo*. During the preparation of this paper a report by Esmon et al. [26] appeared containing similar results with respect to the effects of protein C_a on platelets.

Thrombin may be the physiological activator of protein C. However, Kisiel et al. [3] reported that protein C isolated from serum and plasma were indistinguishable and that fluorescein isothiocyanate-labelled protein C was not activated during clotting of plasma *in vitro*. But their experiments do not rule out local activation of protein C during blood coagulation *in vivo*. In this context it is noteworthy that protein C is activated during prothrombin activation by factor X_a in the presence of isolated platelets, although at a relatively low rate. The amount of protein C activated within 20 min is sufficient to destroy the factor X_a receptors after short incubation. The relatively low activation rate may suggest that a cofactor is missing or that the low rate is physiologically significant, i.e. if protein C is activated too early it will interrupt the prothrombin activation prematurely. Our experimental set up does not simulate the conditions *in vivo*, e.g. a vascular injury results in a local platelet concentration exceeding by far that in our

test-tube experiments. High platelet concentrations result in high activation rates and the local thrombin generation *in vivo* is probably higher than that formed in our experiments. The specific destruction, by protein C_a, of factor V_a either as a part of the platelet factor X_a receptor or emanating from plasma lends support to the assumption that protein C_a plays a role as regulator of the prothrombin activation *in vivo*.

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Human Coagulation Factor V Purification and Thrombin-catalyzed Activation

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ABSTRACT Factor V was isolated from human plasma by barium citrate adsorption, polyethylene glycol fractionation, DEAE-Sepharose CL-6B chromatography, ammonium sulfate fractionation, and gel chromatography on Ultrogel 22. Degradation of Factor V during purification was largely prevented by ample use of inhibitors of proteolytic enzyme. The purified Factor V was a stable, single-chain molecule with an apparent molecular weight of 330,000. Activation of human Factor V by thrombin resulted in a 10- to 15-fold increase in activity. The activation pattern as monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis was compared with that of bovine Factor V. Differences in the patterns of thrombin activation were noticed between the two species, whereas the final products were similar. The products of human Factor V activation are two closely spaced doublets, one with an apparent molecular weight of ~110,000, and the other, ~72,000. An antibody was raised against the purified protein. Crossed immunoelectrophoresis showed that the antibody recognized Factor V both before and after activation with thrombin.

INTRODUCTION

Factor V is a high molecular weight plasma protein. It is an essential, nonenzymatic, component of the prothrombinase complex, which also comprises phospholipid, calcium ions, and the serine protease Factor X_a (1). Factor V also occurs in platelets and is released when platelets are stimulated by thrombin, for example (2, 3). When released from the platelets it binds to the platelet surface, forming part of the binding site for Factor X_a (4-8).

Bovine Factor V is more stable than its human counterpart, for which reason most of our knowledge derives from studies using bovine material (9-14). However, only recently was bovine Factor V purified to

homogeneity, by Nesheim et al. (12) and by Esmon (13). These investigators stressed the importance of using quick separation techniques and of the addition of abundant protease inhibitors throughout the purification procedure. The Factor V obtained was a single-chain protein with a molecular weight of 330,000 (12). Well-defined proteolytic cleavages with concomitant increase in Factor V activity occurred on incubation with a catalytic amount of thrombin (13, 14).

Human Factor V is reportedly extremely labile and only a few attempts have been made to isolate it (9). In 1975, Rosenberg et al. (15) described a four-step procedure including isoelectric precipitation, hydroxylapatite batch adsorption and elution, polyethylene glycol fractionation, and DEAE-cellulose chromatography. The isolated protein gave a single band on isofocusing gel electrophoresis but was not characterized by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. When incubated with thrombin, the Factor V activity increased only twofold to threefold, suggesting that the final product was partially degraded. More recently, Bolhuis et al. (16) described a procedure including cryoprecipitation, polyethylene glycol fractionation, gel filtration on Ultrogel 44 (LKB Produkter AB, Bromma, Sweden), and adsorption of contaminating haptoglobin to immobilized hemoglobin. It was, however, not unambiguously demonstrated that the isolated protein was indeed Factor V, e.g., that the protein was cleaved by thrombin quantitatively. In our laboratory the procedure described has not yielded a pure Factor V preparation.

This paper reports a simple method for isolating a stable, apparently undegraded form of human Factor V. The molecular weight of the isolated protein was identical with that of bovine Factor V. The activation of human Factor V by thrombin resulted in a 10- to 15-fold increase in Factor V activity. The activation products were characterized by SDS-polyacrylamide gel electrophoresis. An antibody against human Factor V was also raised.

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METHODS

DEAE-Sepharose CL-6B was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Ultrogel 22 was from LKB Produkter AB, Bromma, Sweden, and agarose from Marine Colloids Inc., Springfield, N. J. Bovine serum albumin, soybean trypsin inhibitor, phenylmethanesulfonyl fluoride (PMSF)¹ and diisopropylphosphofluoridate (DFP) were from Sigma Chemical Co., St. Louis, Mo. Ortho brain thromboplastin was from Ortho Diagnostics, Inc., Raritan, N. J. Polyethylene glycol (PEG), with the mean molecular weight of 6,000, (PEG-6,000) was from Kebo-Grave AB, Malmö, Sweden.

Bovine as well as human thrombin was prepared essentially according to Lundblad et al. (17). They had fibrinogen clotting activities of 2,500 and 2,300 National Institutes of Health U/mg, respectively, when tested according to Fenton and Fasco (18).

Factor V assay

Factor V-deficient plasma was prepared as described (19). Factor V activity was determined by the method of Kappeler (20). The assay was performed in a Fibrometer Coagulation Timer (BBL Microbiology Systems, Becton, Dickinson & Co., Cockeysville, Md.). The sample (0.1 ml) was incubated with 0.1 ml Factor V-deficient plasma and 0.1 ml brain thromboplastin at 37°C. After exactly 30 s, 0.1 ml 25 mM calcium chloride was added and the clotting time measured. Samples were diluted in 0.036 M sodium acetate, 0.036 M sodium barbitone, 0.14 M NaCl pH 7.4, containing 1% bovine serum albumin. Pooled human citrated plasma, stored in aliquots at -70°C, was used as a source of Factor V in the preparation of standard curves. 1 U of Factor V was defined as the activity found in 1 ml of the pooled plasma.

Antisera

Antisera against the purified Factor V were raised in rabbits. Approximately 100 µg of protein was diluted to a final volume of 1 ml in 0.9% NaCl and emulsified in Freund's complete adjuvant. Rabbits were immunized by subcutaneous injections, and booster doses with the same amount of antigen were given every 3rd wk until the response was satisfactory. Monospecific antisera against human albumin and prothrombin were available at the laboratory.

Electrophoretic and immunochemical methods

Agarose gel electrophoresis was run at pH 8.6 in 0.075 M barbital buffer containing 2 mM calcium lactate (21). Crossed immunoelectrophoresis and electroimmunoassay were performed as described (22, 23). SDS-polyacrylamide disc gel electrophoresis was done in gels containing 5% acrylamide (24).

Amino acid analysis

The amino acid composition of human Factor V was determined in acid hydrolysates (24 and 72 h in 6 M HCl at 110°C *in vacuo*) with standard procedures using a single-column program on a Kontron amino acid analyzer with a Durrum resin

(DC4A, Durrum Chemical Corp., Palo Alto, Calif.). Half-cystine was determined as cysteic acid after performic acid oxidation (25).

Purification of Factor V

All manipulations of the samples were performed on an ice bath; chromatographies and centrifugations were run at 4°C.

Blood collection. Human plasma was obtained from the local blood bank. Approximately 400 ml blood was collected in 63 ml CPD-adenine (Travenol Laboratories, Inc., Deerfield, Ill.). After separation of blood cells by centrifugation at 5,000 g for 10 min the plasma was kept at 4°C for at most 1 h before the purification of Factor V was started. Freshly frozen plasma (-70°C) was occasionally used as starting material. The following protease inhibitors were added to the plasma: benzamidine hydrochloride (1 mM), soybean trypsin inhibitor (50 mg/liter), DFP (0.5 mM), and PMSF (0.5 mM).

Barium citrate adsorption. 80 ml of 1 M BaCl₂ was added dropwise per liter of plasma. After the mixture had been stirred for 1 h, the barium citrate was removed by centrifugation at 6,000 g for 10 min.

PEG-6,000 fractionation. To the supernate from the previous step solid PEG-6,000 was added (80 g/liter). After stirring for 1 h the precipitate was removed by centrifugation at 6,000 g for 10 min. 40 g of solid PEG-6,000 was then added per liter of supernate. The solution was stirred for 1 h, after which the precipitate was collected by centrifugation at 6,000 g for 10 min and the supernate was rejected.

DEAE-Sepharose chromatography. The PEG-precipitated material was dissolved in 200 ml ice-cold 50 mM Tris-HCl, 0.1 M NH₄Cl pH 7.5 containing benzamidine hydrochloride (1 mM), DFP (1 mM), PMSF (1 mM), and 10 mg soybean trypsin inhibitor. The dissolved pellet was deposited on a column (5 × 20 cm) of DEAE-Sepharose CL-6B (Fig. 1) Factor V

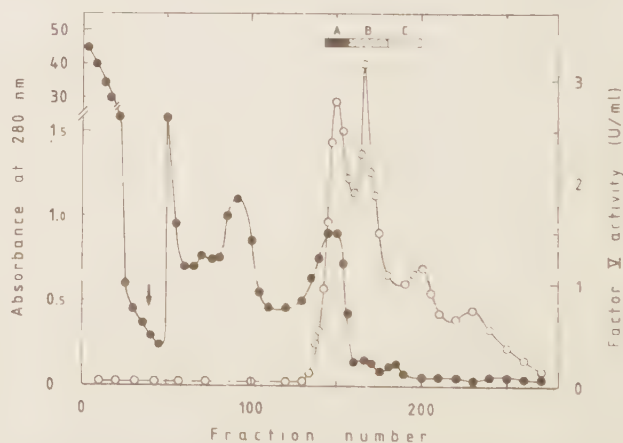


FIGURE 1 DEAE-Sepharose CL-6B chromatography of the 8-12% PEG-6,000 fraction. The column (5 × 20 cm) was equilibrated in 50 mM Tris-HCl, 0.1 M NH₄Cl pH 7.5, and 1 mM benzamidine hydrochloride. After application of the sample, the column was washed with the equilibration buffer at a rate of 150 ml/h for 5-6 h (only the last part of the breakthrough protein peak is shown). Factor V was then eluted with a linear gradient of NH₄Cl (0.1-0.35, 1.15 liter per vessel) in the equilibration buffer containing 10 mM calcium chloride. The flow rate was 150 ml/h and 10-ml fractions were collected. ●, absorbance at 280 nm; ○, Factor V activity. The arrow indicates where the gradient was started. Fractions were pooled as indicated by the horizontal bars.

¹ Abbreviations used in this paper: DFP, diisopropylphosphofluoridate; PEG, polyethylene glycol; PEG-6,000, polyethylene glycol (6,000 mol wt); PMSF, phenylmethanesulfonyl fluoride.

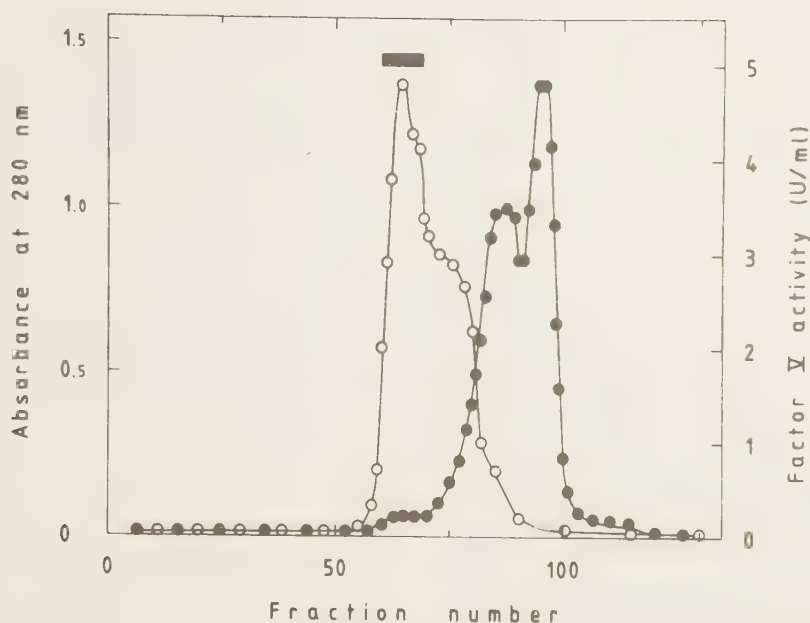


FIGURE 2 Gel chromatography on Ultrogel 22. The ammonium sulfate-precipitated DEAE-Sephacrose pools A to C were separately applied to a column (1.6×91 , 1.6×84 , and 1.6×84 cm, respectively) with Ultrogel 22 in 50 mM Tris-HCl (pH 7.5), 0.1 M NH_4Cl , 10 mM calcium chloride, and 1 mM benzamidine hydrochloride. Fractions of 1.43 ml were collected at a flow rate of 5.7 ml/h. The figure shows the chromatography of pool A (Ultrogel A). ●, absorbance at 280 nm; ○, Factor V activity. Fractions were pooled as indicated by the horizontal bar.

activity was measured, pooled fractions were kept on an ice bath, and Factor V was precipitated by solid ammonium sulfate (60% saturation).

Gel filtration on Ultrogel 22. The precipitates from the previous step were collected by centrifugation at 10,000 g for 15 min and dissolved in a minimal volume of 50 mM Tris-HCl, 0.1 M NH_4Cl , 10 mM calcium chloride pH 7.5 containing 1 mM DFP, 1 mM PMSF, and 1 mM benzamidine hydrochloride. The samples were applied to a column of Ultrogel 22 (Fig. 2). The fractions containing Factor V activity were pooled as indicated in the figure and dialyzed overnight on an ice bath against 25 mM Tris-HCl, 0.05 M NH_4Cl , 5 mM calcium chloride pH 7.5 containing 0.5 mM benzamidine hydrochloride, and 50% glycerol. The protein solutions concentrated by the dialysis by a factor of three to four were then stored at -20°C .

Bovine Factor V was prepared essentially with the method described above.² The major difference between the two species was the limit of precipitation by PEG-6,000, which was 5–10% for bovine Factor V. Furthermore, the yield was higher (25–30%). The isolated bovine Factor V was undegraded and homogeneous on SDS–5% polyacrylamide gel electrophoresis.

Activation of Factor V by thrombin

Factor V (0.1–0.3 mg/ml) in 50% glycerol, 25 mM Tris-HCl, 50 mM NH_4Cl pH 7.5, 0.5 mM benzamidine hydrochloride, 5 mM calcium chloride was kept on an ice bath. Bovine or human thrombin (stock solution of 3,000 and 650 U/ml, respectively) was then added to a final concentration of 2.5 U/ml. Samples of the incubate (100–200 μl) were removed at various

intervals and added to an equal volume of 50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol. No further proteolysis of Factor V, as judged by SDS-polyacrylamide gel electrophoresis, could be detected in this buffer. Aliquots were also drawn, diluted 1,000–100,000 times in the Factor V assay buffer and immediately assayed for Factor V activity. After 20 min incubation on an ice bath, the reaction temperature was raised to 37°C to accelerate the activation process, and after another 10–15 min additional thrombin was added (2.5 U/ml).

RESULTS

Comments on the purification of Factor V. Because human Factor V activity in plasma is known to be very labile, probably because of its sensitivity to proteolytic enzymes, the isolation was performed at 0° – 4°C without interruption. Several protease inhibitors were added throughout the purification. The isolation procedure was designed to avoid lengthy dialysis and concentration steps, as summarized in Table I.

Citrated human plasma, kept at 37°C , rapidly lost its Factor V activity, and after 6 h it had ~30% of that originally found, while bovine plasma kept under identical conditions still had ~60–70%. When freshly frozen plasma was used as starting material without added DFP, the final product contained not only undegraded Factor V, but also several lower molecular weight forms representing partially degraded Factor V. The barium citrate adsorption of plasma was included to remove the vitamin K-dependent proteins and reduce the risk of formation of small amounts of

² B. Dahlbäck and J. Stenflo, unpublished results.

TABLE I
Purification of Factor V

Fraction	Volume	Total protein	Total Factor V activity	Specific activity	Purification	Recovery
	ml	Absorbance units at 280 nm	U	U/ml/A ₂₈₀	X-fold	%
Plasma	1,770	101,000	3,800	0.038	1	100
Barium citrate supernate	1,800	100,000	3,000	0.03	0.8	79
8–12% PEG-6,000 precipitate	200	12,720	1,800	0.14	3.7	47
DEAE-Sepharose CL-6B						35
Pool A	148	115	444	3.9	102	
Pool B	250	39	650	16.7	439	
Pool C	208	27	250	9.3	244	
Ultrogel 22						6
A	15	1.05	54	51	1,350	
B	11.5	1.04	104	100	2,630	
C	11.5	0.51	58	114	3,000	

thrombin or other active coagulation factors during subsequent steps. When this step was excluded, the limits of precipitation of Factor V in the following PEG-6,000 fractionation were higher (~12–18% instead of 8–12%) and electroimmunoassay showed a considerable amount of prothrombin (30–50% of that in plasma) in the dissolved precipitate. The PEG-6,000 precipitation gave a four to fivefold purification and rapidly reduced both the volume and the total amount of protein. The dissolved precipitate proved suitable for direct application to the DEAE-Sepharose CL-6B column (Fig. 1). This step was concluded ~16–20 h after the blood had been collected and produced a 30-fold (pool A) to 120-fold (pool B) purification with a recovery of ~60%. Factor V was bound very firmly to the column and was eluted late in the gradient. Factor V precipitated by 60% ammonium sulfate at this stage of purity could be stored at 4°C for weeks without any significant loss of activity. Pools A to C from the DEAE-Sepharose were separately chromatographed on a column with Ultrogel 22 (abbreviated Ultrogel A to C, see Table I). The chromatogram obtained when DEAE-Sepharose pool A was applied to the column with Ultrogel 22 is shown in Fig. 2. DEAE-Sepharose pools B and C gave chromatograms similar to that of pool A. After dialysis against glycerol/ buffer and 4 mo of storage at –20°C no loss of Factor V activity or degradation was noticed, as judged by SDS-polyacrylamide gel electrophoresis. The specific activities in the best Factor V preparations were 50–100 U/ml per absorbance unit at 280 nm, and on incubation with thrombin they were as high as 500–1,500 U/ml per absorbance unit at 280 nm.

Characterization of Factor V by SDS-polyacrylamide gel electrophoresis. The Factor V obtained after chromatography on Ultrogel 22 of DEAE-Sepharose pool A was nearly homogeneous (Fig. 3). Analysis on 10% gels showed no additional protein

bands. The molecular weight of human Factor V was estimated relative to that of bovine Factor V, which is reported to be 330,000 (12). Before and after reduction of disulfide bridges, mixed human and bovine Factor V appeared as a single band on 5% polyacrylamide gel electrophoresis, with SDS indicating also that the molecular weight of human Factor V was 330,000. Factor V obtained after chromatography on Ultrogel 22 of DEAE-Sepharose pools B and C contained several electrophoretic components with lower molecular weight (components B and C, Fig. 3). Like single-chain Factor V, these bands disappeared completely upon activation with a catalytic amount of thrombin,

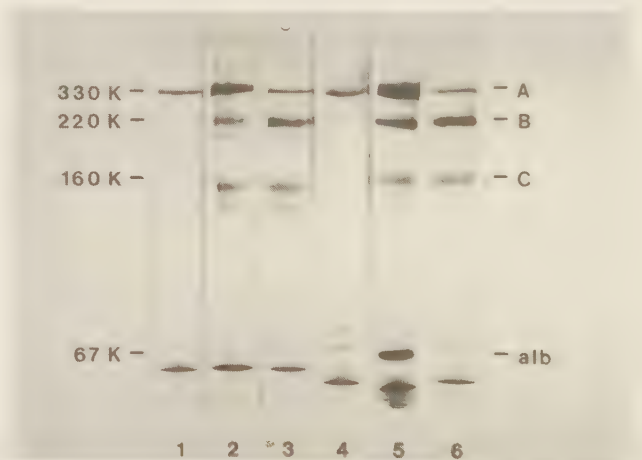


FIGURE 3 SDS-5% polyacrylamide gel electrophoresis of human Factor V obtained from Ultrogel A to C. Samples 1–3 were unreduced and 4–6 reduced. The relative molecular weights of the components are indicated at the left. Samples 1 and 4, from Ultrogel A; samples 2 and 5, Factor V from Ultrogel B; samples 3 and 6, Factor V from Ultrogel C. Approximately 15 µg protein was loaded on gels 1, 3, 4, and 6 and 25–30 µg protein on gels 2 and 5. The tracking dye is seen as a band at the bottom of each gel.

indicating that they represented partially degraded Factor V. In Ultrogel B and C a small amount of Factor V activity was eluted earlier than the main Factor V peak. On SDS-polyacrylamide gels, this material gave a pattern identical with that of the protein of the mean peak in the same column, and probably represented an aggregated form of Factor V.

Occasionally the Factor V preparation contained a contaminant, which was rendered on reduced gels only (component alb in Fig. 3), and had an apparent molecular weight of 66,000 after reduction of disulfide bridges. On agarose gel electrophoresis it migrated like albumin, to which it is also immunochemically related. It was probably albumin molecules linked together by disulfide bridges. In most preparations this polymeric albumin was not visible, and in no other preparation was it as obvious as in Factor V from Ultrogel B, shown in Fig. 3.

Amino acid composition. The amino acid composition of human Factor V is shown in Table II, together with the composition reported for bovine Factor V. The composition of our bovine Factor V preparation (not shown) was in good agreement with that reported by Nesheim et al. (12). The carbohydrate content of human Factor V was assumed to be identical to that of bovine Factor V (12), which gives a molecular weight of the apoprotein of 279,000. The major difference in amino acid composition between the two species was the

higher half-cystine content in human Factor V. This difference was found in two preparations.

Activation of Factor V by thrombin. The conclusion that the products of the purification procedure were indeed Factor V was based on the high specific activity of the protein and on the finding that activation with both human and bovine thrombin resulted in complete conversion of the protein to lower molecular weight products. Catalytic amounts of thrombin were used, and the reaction mixtures were kept on an ice bath to facilitate identification of early activation intermediates. After 10 min the temperature was increased to 37°C, and after another 10 min an additional aliquot of thrombin was added. The Factor V activity was followed and the reaction products were examined with SDS-polyacrylamide gel electrophoresis (Fig. 4). The pattern obtained was compared with that of thrombin-catalyzed activation of bovine Factor V, and the nomenclature of Nesheim and Mann (14) was used. Activation of human Factor V by human and by bovine thrombin gave identical patterns on SDS-polyacrylamide gels. The high molecular weight form of Factor V (component A) was quickly cleaved even at the low initial temperature, and electrophoretic components A₁ and D (and to some extent, also component B) were formed with a concomitant fivefold increase in Factor V activity. Component D, which on reduced gels appeared as a closely spaced doublet, was stationary throughout the incubation and appeared to be an end product. After increasing the temperature to 37°C there was an additional twoto threefold increase in activity. Component A₁ (and B) disappeared at the same time as component E and F appeared. The gel pattern was the same when the reaction temperature was 37°C throughout the experiment, although early intermediates were less easily seen.

Thrombin activation of Factor V obtained from the more heterogeneous Ultrogel B fraction was also followed to determine whether the electrophoretic components B and C, which were not easily observed during activation of intact Factor V, were indeed related to Factor V. Both bands B and C disappeared during the activation, and it therefore appears that these components were partially degraded Factor V. During the activation of intact Factor V, electrophoretic component B was seen as a faint band, whereas component C, in our system, could not be demonstrated as an intermediate. After completed activation the patterns were identical whether homogeneous high molecular weight Factor V or partially degraded Factor V was used. The Factor V activities found are also shown in Fig. 4. The increase in Factor V activity during thrombin activation was the same whether Factor V from Ultrogel A or B was used, whereas the more degraded Factor V from Ultrogel C increased only two- to threefold in activity.

TABLE II
Amino Acid Composition of Human Factor V

Amino acid*	Human	Bovine†
Aspartic acid	252	299
Threonine‡	161	121
Serine§	245	204
Glutamic acid	305	293
Proline	144	199
Half-cystine	83	24
Glycine	161	146
Alanine	142	131
Valine	135	108
Methionine	61	43
Isoleucine	102	124
Leucine	252	240
Tyrosine	81	94
Phenylalanine	91	88
Lysine	139	148
Histidine	62	71
Arginine	108	110
Total	2,524	2,443

* Composition expressed as residues per molecule assuming a molecular weight of the apoprotein of 279,000.

† Data from Nesheim et al. (12). The composition of bovine Factor V is shown for comparison.

§ Extrapolated to zero-time hydrolysis.

|| 72-h hydrolysis value.

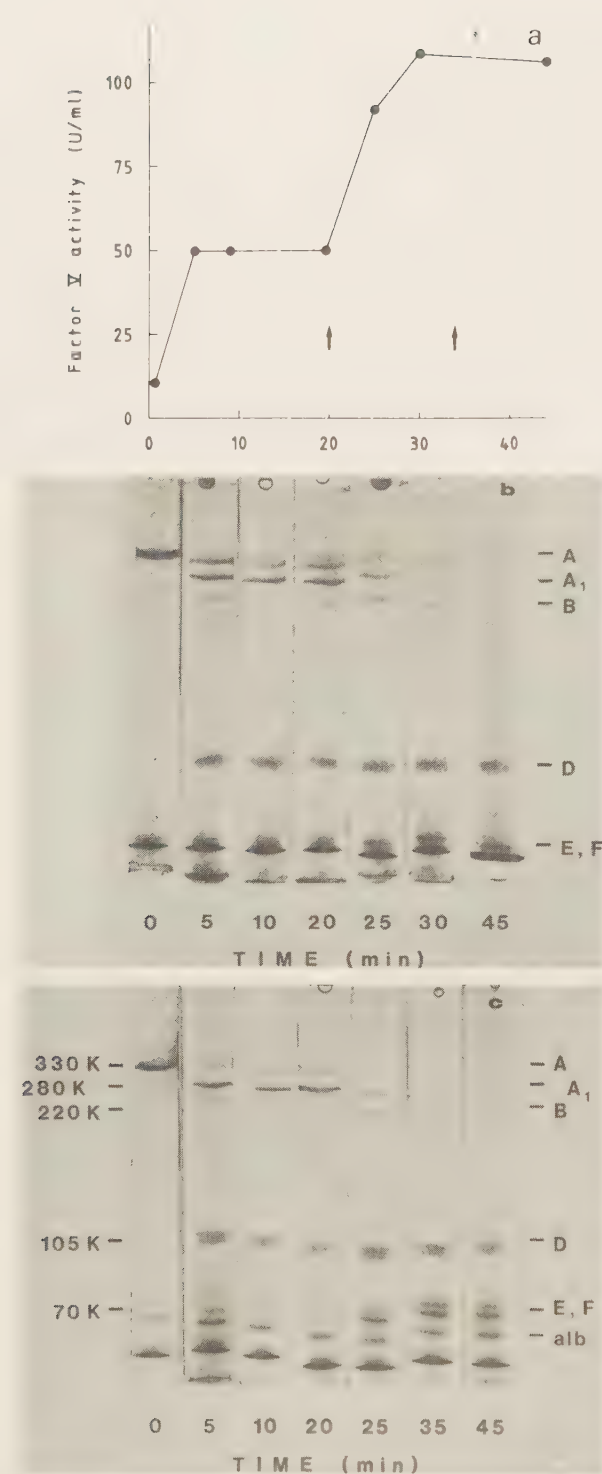


FIGURE 4 Activation of intact human Factor V by thrombin. Human Factor V from Ultrogel A (10 U/ml) was incubated with bovine thrombin (2.5 U/ml). The reaction mixture was kept on an ice bath; after 20 min (first arrow) it was equilibrated at 37°C, and after 34 min (second arrow) an additional aliquot of

Thrombin-catalyzed activation of bovine Factor V was also studied (Fig. 5) to facilitate comparison of the products with those of human Factor V. In the experiment presented in Fig. 5 the reaction temperature was 37°C. No difference in the pattern of thrombin activation could be detected if the reaction mixture initially was kept on an ice bath. Our activation pattern of bovine Factor V seemed identical to those on record (13, 14), and the nomenclature of Nesheim and Mann (14) was adopted. There were distinct differences between the two species in the activation pattern, although the final products were similar. As previously mentioned, the dominating intermediate in human Factor V activation was component A₁, whereas in the activation of bovine Factor V, components B and C were predominant. These components closely resembled the additional electrophoretic components B and C in partially degraded human Factor V. The cause of this difference between the activation of human Factor V in the pure system and of the proteolysis that had occurred during the purification is not clear, but it may be due to cleavage of human Factor V by some protease other than thrombin during purification.

The molecular weight of single-chain bovine Factor V has been determined by Nesheim et al. (14) to 330,000 by sedimentation equilibrium analyses. When the logarithms of this value and the reported molecular weights of the bovine Factor V activation products (14) were plotted against the relative mobilities (on reduced SDS-5% polyacrylamide gels) of the corresponding protein bands, a straight line was obtained. This plot was used as molecular weight standard curve in the determination of the apparent molecular weights of the human components (Table III).

Following activation of human Factor V by thrombin, aliquots were removed and stored at 22°, 4°, and at -20°C to determine the stability of the factor V_a activity. No differences in stability between the aliquots stored at the three temperatures could be noticed. After 24, 48, and 72 h of storage ~70, 30, and 7%, respectively, of the original Factor V_a activity was retained. A parallel aliquot, 20 mM in EDTA and stored at 4°C, retained 30% of its activity after 3 h, 7% after 24 h, and 1% after 48 h, indicating that human Factor V_a, like bovine Factor V_a (13), requires calcium ions to retain its biological activity.

Immunochemical studies. Crossed immunoelec-

thrombin (2.5 U/ml) was added. At the times indicated aliquots were removed and subjected to SDS-5% polyacrylamide gel electrophoresis. (a) Factor V activity, (b) gels with unactivated samples, and (c) reduced samples are shown. Components and relative molecular weights are indicated. Approximately 15 µg protein was loaded on each gel. The tracking dye is seen as a band at the bottom of each gel.

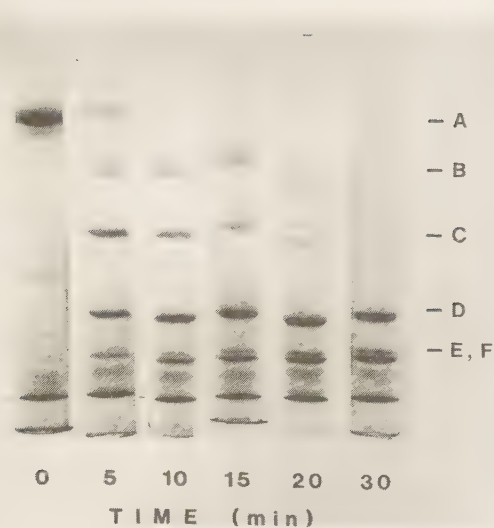


FIGURE 5 SDS-5% polyacrylamide gel analysis of the activation of bovine Factor V by thrombin. Bovine Factor V (40 U/ml) was incubated with bovine thrombin (2.5 U/ml) at 37°C. After 17 min an additional aliquot of thrombin (2.5 U/ml) was added. At various intervals samples were drawn, reduced, and subjected to SDS-5% polyacrylamide gel electrophoresis. Approximately 20 μ g protein was loaded on each gel. The tracking dye is seen as a sharp band at the bottom of each gel.

trophoresis of Factor V was run both before and after activation by thrombin (Fig. 6). Before activation, Factor V migrated to a position just behind the α_1 -antitrypsin band, whereas after activation it migrated more anodally. The surface under the immunopre-

TABLE III
Molecular Weight of Human Factor V
and Its Activation Products

Component	Bovine Factor V		Human Factor V
	Nesheim and Mann	Esmon	
		<i>mol wt</i>	
A	330,000	280,000	330,000
A ₁			280,000
B	205,000	210,000	220,000
C	150,000	Not reported	160,000
D	94,000	115,000	110,000
E	74,000	73,000	74,000
F	71,000		71,000

The apparent molecular weights of human Factor V and its activation products were determined by electrophoresis on 5% SDS-polyacrylamide gels after reduction of disulfide bridges. The bovine Factor V activation products with the molecular weights given by Nesheim and Mann (14) were used to construct the calibration curve. The data of Esmon (13) are also included for comparison.

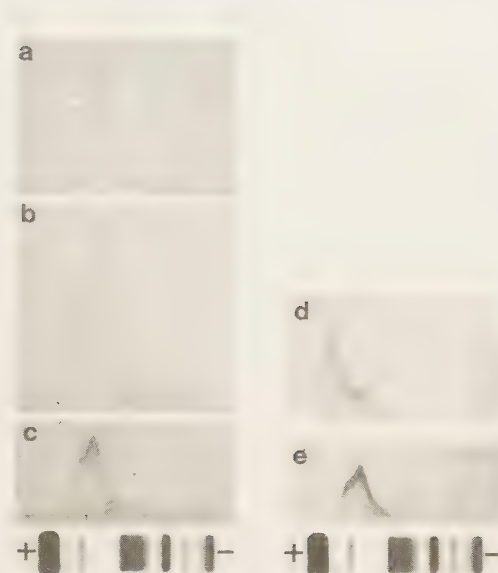


FIGURE 6 Crossed immunoelectrophoresis of human Factor V. Factor V from Ultrogel C (a), Factor V from Ultrogel B (b), Factor V from Ultrogel A (c), and human Factor V from Ultrogel A after (d) and before (e) activation with human thrombin (2.5 U/ml) at 37°C are shown.

cipitate was larger after activation by thrombin, a finding suggesting that the activated protein had lost antigenic determinants. When plasma was analyzed by electroimmunoassay with the antiserum against Factor V, a rocket-like precipitate could clearly be seen, whereas it was faint and barely visible when serum was used.

DISCUSSION

The extreme lability of human Factor V in plasma, used in preparing Factor V-deficient plasma by aging, is the main reason why isolation procedures yielding an undegraded product have not been reported previously (9, 15). The lability can presumably be explained by the high susceptibility of Factor V to proteolysis during blood collection as well as during the initial purification steps. The successful purification of the somewhat more easily handled bovine Factor V (12, 13) drastically improved the prospects of successful purification of human Factor V. The isolation procedure for human Factor V described in this paper uses fresh plasma, several protease inhibitors, and a low temperature (0°–4°C). The initial steps are quick and performed without interruption. The final product is stable for >4

mo. It was also observed that the Factor V activity after the DEAE-Sepharose chromatography was stable for days when stored at 4°C. This indicates that human Factor V, once separated from proteases and their zymogens, is not an intrinsically labile protein.

Though strict precautions were taken to minimize proteolysis, some of the material obtained was partially degraded, probably due to proteolysis during blood collection or the first purification steps. In this context it should perhaps be pointed out that the starting material contained ~2 U of Factor V per milliliter, indicating that when obtained from the blood bank the plasma Factor V was already partly activated. If the local conditions allow human blood to be collected directly into anticoagulant containing protease inhibitors, the final product will probably contain a smaller amount of degraded Factor V. The partially proteolyzed material was eluted from the ionic exchange column at higher salt concentration than undegraded Factor V, as has also been reported for bovine Factor V (13).

The final yield of the purification was low. This may have been caused in part by premature activation of Factor V and rapid subsequent loss of activity. When bovine Factor V was isolated with essentially the same procedure, the yield was higher and comparable to those previously reported (12, 13). Furthermore, there were no partially degraded forms of the bovine factor.

The activation of human Factor V, followed by SDS-polyacrylamide gel electrophoresis, differed from that of bovine Factor V. Thus, component A₁ could not be demonstrated on bovine Factor V activation; and component C, although present in the partially degraded human Factor V, was apparently not an intermediate in the activation of human Factor V in our system. The final activated products were, however, similar. Human component D, which on reduced SDS-polyacrylamide gels appeared as a doublet, has a somewhat higher molecular weight (110,000) than bovine component D (94,000) (12), whereas human and bovine components E and F have virtually identical molecular weights (74,000 and 71,000, respectively).

The possibility of isolating human Factor V will advance our understanding of the role played by human Factor V in the prothrombinase complex and as part of the platelet Factor X_a receptor. Furthermore, the availability of a monospecific antibody will be useful in the development of methods to supplement the conventional clotting assay in the evaluation of various disorders affecting the coagulation system.

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